University of Alberta

THE CONTRIBUTION OF β_3 INTEGRIN-MEDIATED COSTIMULATION TO T CELL ACTIVATION

by

EILEEN ALICE MA

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

Department of Medical Microbiology and Immunology

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **"THE CONTRIBUTION OF β_3 INTEGRIN-MEDIATED COSTIMULATION TO T CELL ACTIVATION"** submitted by **EILEEN ALICE MA** in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**.

Hanne Ostergaard

Linda Pilarski

Andrew Shaw

July 2, 1996

DEDICATION

I would like to dedicate this thesis to the memory of my father

Dr. John Wing-Sang Ma

He will always be an inspiration to me, and I love him dearly.

ABSTRACT

T cell activation involves many cell surface antigens, including the antigen-specific T cell receptor and several accessory molecules, that induce a cascade of signaling events and results in effector function. Accessory molecules expressed on the T cell surface function to enhance adhesion, amplify signals, or transduce distinct signals from the T cell receptor complex. Integrins, a family of extracelilular matrix protein and cell surface protein receptors, mediate adhesion and have been shown to costimulate T cell activation of $CD4^+$ T cells in response to TCR or CD3 stimulation.

We examined $CD8^+$ cytotoxic T cells for their ability to utilize ECM proteins as costimulatory molecules in T cell activation and identified an integrin receptor that was mediating the responses. We looked at early signaling events and proteins altered by costimulation to try and identify the signaling pathways induced by the ECM proteins.

We found that fibronectin, vitronectin, and fibrinogen, but not collagen or laminin, co-immobilized with sub-stimulatory anti-CD3, could facilitate degranulation and induce tyrosine phosphorylation of 115-125 kDa proteins, with a 116 kDa protein being the most prominent phosphoprotein, in cytotoxic T cell clones. The responses were inhibited by RGD peptides.

The specificity of the responses implicated a β_3 integrin, in particular the vitronectin receptor ($\alpha_v\beta_3$), in mediating the signal transduction and as an accessory molecule in CTL. Consistent with this hypothesis, we demonstrated

expression of β_3 integrin by FACS analysis and immunoprecipitation, and engagement of β_3 integrin with immobilized antibody to β_3 stimulated tyrosine phosphorylation of the 115-125 kDa proteins. Soluble β_3 antibodies could effectively inhibit fibronectin- and vitronectin-stimulated phosphorylation, and phosphorylation was also blocked by cytochalasins, indicating an intact cytoskeleton was required for phosphorylation. We were able to immunoprecipitate a 116 kDa protein, recognized by a $pp125^{FAK}$ antibody, that became phosphorylated with VN or immobilized anti-β_3 stimulation.

These results demonstrated that CTL express and use a β_3 integrin as an accessory molecule capable of signal transduction which can augment TCR-mediated stimulation in T cell activation.

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TABLE OF CONTENTS

CHAPTER		PAGE
I.	**Introduction**	**1**
	T Cell Activation and Accessory Molecules	1
	The Extracellular Matrix	4
	Integrins	5
	Focal Adhesions	7
	Integrins and Signal Transduction	8
	pp125^{FAK} - Focal Adhesion Kinase	9
	Signaling in Focal Adhesions	11
	Integrins on T Lymphocytes	14
	Integrins as Accessory Molecules in T Cell Activation	15
	Purpose of this Study	19
II.	**Materials and Methods**	**20**
	Cells	20
	Antibodies	21
	Protein Immobilization	22
	CTL Degranulation Assay	22
	Anti-Phosphotyrosine Immunoblotting	23
	FACScan Analysis	23
	Surface Biotinylation and Immunoprecipitation	24
	Immunoprecipitation from Stimulated Cells	25

III. Results 26

Fibronectin is a Costimulator for T Cell Activation 26

FN-Stimulated Responses are RGD-dependent 28

FN, VN, and FG can Stimulate T Cells 34

A β_3 Integrin is Expressed on T Cells 35

β_3 Integrin Antibody can Stimulate Tyrosine Phosphorylation 36

Identification of the Tyrosine Phosphorylated Proteins 49

Involvement of the Fyn and Lck Tyrosine Kinases 50

Possible Regulation of β_3 Integrin 51

An Intact Cytoskeleton is Required 52

IV. Discussion 59

A β_3 Integrin Mediates Costimulation 59

Differences with Previous Studies 60

FN has a Dual Role 62

Possible Role of the FNR 63

Tyrosine Phosphorylation of a pp125^{FAK}-Reactive Protein 64

Tyrosine Phosphorylation of Additional Proteins 65

Tyrosine Kinases are Activated and Mediate Phosphorylation . . . 66

Role of the Cytoskeleton 66

An Elaborate Integrin System 67

V. Conclusions and Future Directions 68

VI. Bibliography 71

LIST OF FIGURES

Figure	Description	Page
1	FN enhancement of anti-CD3-stimulated degranulation of CTL clone 11	29
2	Synergistic enhancement of tyrosine phosphorylation events with FN and anti-TCR in CTL clone 11	30
3	FN-stimulated tyrosine phosphorylation of a 116 kDa protein; Enhanced and prolonged tyrosine phosphorylation events induced synergistically by FN and anti-CD3	31
4	RGD inhibition of FN-enhanced degranulation and tyrosine phosphorylation of pp116 in CTL clone 11	32
5	RGDSP, but not RGDNP, peptides inhibit degranulation of CTL clone 11 stimulated by FN co-immobilized with sub-stimulatory anti-CD3	33
6	Anti-CD3-stimulated degranulation of CTL clone 11 facilitated by FN, VN, and FG	38
7	Anti-CD3 stimulated degranulation of CTL clone AB.1 facilitated by ECM proteins	39
8	Tyrosine phosphorylation events in CTL clone 11 induced by ECM proteins in the presence and absence of anti-CD3	40
9	Tyrosine phosphorylation events in CTL clone AB.1 induced by ECM proteins in the presence and absence anti-CD3	41
10	Tyrosine phosphorylation events induced by ECM proteins in the presence and absence of anti-CD3 in $CD4^+$ T_H clones	42

11	Tyrosine phosphorylation events in Con A activated splenic T cell blasts induced by ECM proteins in the presence and absence of anti-CD3	43
12	FACS analysis of the expression of integrins on CTL clone 11	44
13	Immunoprecipitation of integrin subunits from surface biotinylated CTL clone 11	45
14	Integrin antibody stimulation of tyrosine phosphorylation	46
15	Antibody inhibition of VN- and FN-induced tyrosine phosphorylation	47
16	Antibody inhibition of VN- and FN-facilitated degranulation stimulated by anti-CD3	48
17	Immunoprecipitation of a pp125^{FAK}-reactive protein phosphorylated by stimulation with VN or β_3 integrin antibody	53
18	C-cbl is present but not tyrosine phosphorylated by immobilized β_3 antibody in CTL clone 11	54
19	Induction of tyrosine phosphorylation in lck-deficient and fyn-deficient CTL clones	55
20	Examination of the tyrosine phosphorylation and coimmunoprecipitated proteins of β_3 integrin	56
21	Cytochalasin D inhibition of tyrosine phosphorylation induced by FN or VN in the presence and absence of anti-CD3	57
22	Cytochalasin E inhibition of tyrosine phosphorylation induced by FN or VN in the presence and absence of anti-CD3	58

ABBREVIATIONS

APC - antigen presenting cell

BSA - bovine serum albumin

CAKβ - cell adhesion kinase β

cDNA - complementary DNA

COL I - collagen type I

COL III - collagen type III

Con A - Concanavalin A

CTL - T cytotoxic cell

cyto D - cytochalasin D

cyto E - cytochalasin E

DETC - dendritic epidermal T cell

D-PBS - Dulbecco's phosphate buffered saline

ECM - extracellular matrix

FACS - fluorescence activated cell sorting

FAK - focal adhesion kinase

FAT- focal adhesion targeting

FCS - fetal calf serum

FG - fibrinogen

FITC - flourescein isothiocyanate

FN - fibronectin

FNR - fibronectin receptor

G protein - GTP-binding protein

Grb2 - growth factor receptor-bound protein 2

ICAM-1 - intercellular adhesion molecule-1

Ig - Immunoglobulin

ITAM - immunoreceptor tyrosine-based activation motif

kDa - kiloDalton

LAM - laminin

LFA-1 - leukocyte function-associated molecule-1

mAb - monoclonal antibody

MAP - mitogen-activated protein

MHC - major histocompatability complex

NPXY - asparagine-proline-any amino acid-tyrosine

NP40 - Nonidet P40

PBL - peripheral blood lymphocytes

PBS - phosphate buffered saline

PYK2 - proline-rich tyrosine kinase 2

RAFTK - related adhesion focal tyrosine kinase

RGD - arginine-glycine-aspartic acid

RGE - arginine-glycine-glutamic acid

SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SH - src homology

Sos - son-of-sevenless

TCR - T cell receptor

T_H - T helper

VCAM-1 - vascular cell adhesion molecule-1

VLA - very late activation antigen

VN - vitronectin

VNR - vitronectin receptor

ZAP-70 - zeta chain-associated protein tyrosine kinase-70

CHAPTER I.

INTRODUCTION

Adhesive interactions between a number of different cell types and the meshwork of extracellular matrix (ECM) proteins play critical roles in migration, proliferation, and differentiation. T lymphocytes are capable of cell to cell and cell to ECM interactions and rely on these for adhesion, trafficking, development, activation, and effector function. If T cell recognition of a foreign antigen occurs, adhesion to the antigen presenting cell must be specific and stable for sufficient time to allow effector mechanisms to be completed. Lymphocytes are very motile cells whose efficient response depends on the ability to migrate through the lymphoid system and into tissues. The cell surface molecules which mediate the interactions with ECM are the integrin family of cell surface receptors. Integrins function in adhesion and migration of lymphocytes, and they have an emerging role in signal transduction and activation.

T Cell Activation and Accessory Molecules

When T cells are not participating in an immune response, they are in a resting state. If a T cell encounters a foreign antigen and receives the appropriate stimulatory signals, a cascade of biochemical events is initiated which leads to activation of the cell. The earliest detectable event is tyrosine phosphorylation of a number of cytoplasmic proteins and activation of protein tyrosine kinases. Some phosphorylated proteins have been identified, but there are several which have not been identified. The zeta (ζ)-chains and CD3 of the T cell antigen receptor (TCR)

complex and phospholipase C are proteins phosphorylated upon TCR engagement[1]. The sequence motifs of the ζ and CD3 chains which contain a tyrosine residue that can be phosphorylated are called ITAMs or immunoreceptor tyrosine-based activation motifs. First described by Reth[2], there are three ITAMs in each ζ chain and one in each of the CD3 chains that are responsible for the signal transduction capabilities of the TCR complex[3,4,5]. Lck and fyn, two src-related tyrosine kinases, and ζ chain-associated protein tyrosine kinase-70 (ZAP-70) are involved in this early signaling[6]. ZAP-70 and fyn have been demonstrated to associate with the ITAMs of the ζ chain upon TCR engagement[6].

Another early biochemical event is a rapid change in cytoplasmic calcium ion concentration. Phosphorylation and activation of phospholipase C leads to hydrolysis of phosphoinositides and generation of second messengers, diacylglycerol and inositol trisphosphate[1]. There is also evidence for the involvement of heterotrimeric GTP-binding proteins (G proteins) in T cell activation[7]. Once activated, a T cell can perform its effector function. In the case of a T helper cell (T_H), it will proliferate, secrete cytokines, and help other branches of the immune system. An activated T cytotoxic cell (CTL) is able to lyse an infected target cell by a variety of mechanisms, including releasing its cytotoxic granule contents.

The antigen specificity of a T cell interaction is provided by the TCR complex interacting with an antigenic peptide presented in the groove of the major histocompatibility complex (MHC). In general, MHC Class II on an antigen presenting cell (APC) presents to $CD4^+$ T helper cells (T_H), and MHC Class I on an infected target cell presents to $CD8^+$ T cytotoxic cells (CTL). The binding of TCR

and MHC is necessary but insufficient for activation and alone may lead to an anergic state[8]. Additional cell surface interactions must be engaged to provide necessary adhesive or stimulatory signals for activation. These accessory molecules are numerous and diverse in their costimulation. Accessory molecules can function to enhance the adhesiveness between a T cell and an APC or a target cell by bringing more cell surface area into contact, increasing TCR occupancy, and stabilizing the conjugation. A different contribution of accessory molecules is the ability to amplify the TCR signaling events by enhancing the same signaling pathways as the TCR such that the combination of signals is above threshold and stimulatory for activation. An accessory molecule may also activate distinct signal transduction pathways from the TCR that are important for a complete activatory event.

Some accessory molecules in T cell activation have been characterized that function in at least one of the above described capacities. Leukocyte function-associated molecule-1 (LFA-1) and intercellular adhesion molecule-1 (ICAM-1) interaction is increased in avidity after TCR engagement and strengthens the adhesion between cells[9]. CD4 and CD8 are coreceptors of the TCR complex in that they bind to non-polymorphic regions of MHC molecules and appear to be required for T cell activation[10]. The CD28 of T cells and B7 of APCs interaction is an important costimulator and transduces a different signal from the TCR[11]. As discussed in more detail later, it is clear that T cell interactions with the ECM are also costimulatory in T cell activation.

The Extracellular Matrix

The extracellular matrix is an intricate network of macromolecules found between cells in tissues and proteins of the ECM can be found as a soluble serum components. ECM serves as scaffolding to which cells can anchor, to stabilize the structure and integrity of tissues and support the architecture of an organ. Adhesion to ECM can also determine the developmental fate of a cell. Immature cortical thymocytes, but not mature single $CD4^+$ or $CD8^+$ thymocytes, express receptors for the ECM protein fibronectin (FN)[12,13]. As well, ECM provides a substrate for cells migrating through tissues. For example, lymphocytes may have to exit the circulation and traffic through the tissue matrix to reach their site of action, potentially an inflammatory site. As a cell maneuvers through tissue, it encounters the ECM and relies on it to act as a substrate for motility. In addition to its structural role, cellular interactions with ECM can influence migration, development, proliferation, and function.

A number of proteins and polysaccharides compose the ECM, including FN, vitronectin (VN), collagen, and laminin. Fibronectin[14] is a ubiquitous glycoprotein expressed on cell surfaces and in tissues in an insoluble form and in a soluble form in the plasma. It is secreted as a dimer of 220-250 kDa monomers joined by two disulphide bonds near the carboxyl terminus of the protein. The FN molecule is comprised of several functional domains that bind other ECM, ECM receptors, and blood proteins. Several isoforms of FN exist as a result of alternative splicing. Vitronectin is primarily a serum protein, but it can also be found in tissues and at the cell surface[15]. It is found in wounded tissues and has a role in cell spreading, differentiation, and tumor metastasis[16].

Integrins

Integrins[17] are a large class of cell surface molecules that serve as receptors for ECM proteins, as well as cell surface expressed adhesion molecules such as ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1). Integrins are composed of non-covalently associated heterodimers of an α subunit chain and a β subunit chain. The individual subunits have a large glycosylated extracellular domain, a single transmembrane region, and a short cytoplasmic tail. The α subunits vary in size between 120 to 200 kDa, and the β subunits are from 90 to 140 kDa. In vertebrates, there are currently 15 known α subunits and 8 known β subunits which associate in various, yet restricted, combinations to form 20 known integrins. A given α chain may form a heterodimer with several different β subunits. Families are generally characterized by the β chain in the heterodimer. For example, there are β_1, β_2, β_3 and so on integrin families. There is high conservation of β_1 integrins across species, and 44 to 47 percent similarity between the β_1, β_2, and β_3 subunits[18].

The extracellular domain of the α chain confers most of the ligand specificity, but both subunits are required for its function[17]. Ligand binding is dependent on extracellular divalent cations, either Ca^{2+} or Mg^{2+} depending on the integrin. There are three or four divalent cation binding sites found on the α subunit[17]. Specificity varies among the integrin heterodimers and can differ for a particular integrin depending on the cell type expressing the integrin. There is also developmentally regulated expression of some integrins[13]. Additional diversity is generated through alternative splicing in the cytoplasmic domains of some integrin RNAs, including β_1 and β_3, and this can also alter specificity and/or function[19,20].

There is redundancy in the specificities of the integrins for ECM proteins, in that one particular ECM protein may be recognized by several integrins. For example, fibronectin can be bound by at least nine integrins, including $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_1$, and $\alpha_v\beta_3$, and vitronectin can be bound by at least six integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_{IIb}\beta_3$[21]. Whereas some integrins have a single specificity, such as the integrin $\alpha_5\beta_1$, the classical fibronectin receptor (FNR), many integrins have multiple specificities, such as $\alpha_v\beta_3$, the vitronectin receptor (VNR), for fibronectin, vitronectin, fibrinogen, von Willebrand factor, thrombospondin, and perhaps additional ECM proteins. Determination of integrin specificities, thus, is complicated by multiple factors.

Some integrins exhibit the requirement to be activated in order to bind their ligands. Integrin $\alpha_{IIb}\beta_3$ on resting platelets does not bind its ligands. Only after platelet activation is there a conformational change of the integrin to a high affinity binding state for its ligands[22]. The leukocyte β_2 integrins also exhibit modulation of binding affinity. As with the platelet integrins, upon leukocyte activation, the β_2 integrins undergo a conformational change to activate binding[23]. Furthermore, activation of T lymphocytes enhances the ligand binding of β_1 integrins[24]. This phenomenon is sometimes referred to as "inside-out" signaling where an activation signal transmitted into the cells results in a signal to the integrin inducing a conformational change that allows high affinity integrin binding of ligand[17].

The first characterized recognition site found in many ECM proteins that is bound by several integrins receptors is the tripeptide sequence of L-arginyl-glycyl-L-aspartic acid or Arg-Gly-Asp or RGD[25]. This sequence is present in fibronectin, vitronectin, and a variety of other ECM proteins. The integrins $\alpha_5\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_v\beta_1$,

$\alpha_v\beta_3$, and $\alpha_v\beta_5$ recognize this sequence, and it probably reflects the close relation of the β_1 and β_3 subunits[17]. Binding of integrins to ECM proteins via this sequence can be specifically inhibited by synthetic peptides containing the RGD sequence[26]. Alternatively, integrins may bind ECM proteins through different regions of FN. For example, integrin $\alpha_4\beta_1$ recognizes the tripeptide Leu-Asp-Val of the CS-1 region within the alternatively spliced IIICS domain of fibronectin, and $\alpha_4\beta_1$ can also bind to specific immunoglobulin-like domains of its other ligand, VCAM-1[27]. There are additional recognition sequences that have been determined for fibrinogen and laminin[17].

Integrins are found in most tissues, on both lymphoid and non-lymphoid cells. Some integrins are widespread throughout many tissues, whereas others have very limited distribution. β_1 integrins can be found on most cells, including lymphocytes, and are capable of binding to fibronectin, collagen, and laminin. The β_2 integrin family, though, is restricted to leukocytes and is often referred to as the leukocyte integrins. The β_3 integrins are expressed on platelets in association with α_{IIb}, and when coupled with α_v represent the vitronectin receptor and are found on inflammatory cells, tumor cells, and subsets of lymphocytes[16,28].

Focal Adhesions

Focal adhesions are the sites of close and tight physical contact between a cell and the extracellular milieu. Components of a focal adhesion include ECM proteins in the insoluble matrix or expressed on a cell surface, integrin receptors, and cytoskeletal proteins. ECM proteins, such as FN and VN, are bound by the cell through integrin receptors and linked on the cytoplasmic side of the junction to

several cytoskeletal proteins. Vinculin, talin, and paxillin are present, as well as actin-binding proteins such as α-actinin and tensin. Actin stress fibers terminate in focal adhesions. By definition, focal adhesions are areas where the internal cytoskeleton of the cell is linked to the external environment through integrins[29].

Besides structural proteins, there are regulatory proteins localized to focal adhesions[29]. They include signaling molecules such as kinases and may regulate formation and disassembly of focal adhesions. As will be discussed later, these regulatory proteins also apparently have roles in regulating cell growth.

Integrins and Signal Transduction

As well as their function in adhesion to ECM, it has been demonstrated in several non-hematopoetic cell types that integrins can have an additional role important in cell growth[17,30,31,32] and apoptosis[33]. Integrin engagement can induce many signal transduction pathways, and the biochemical events which occur have aided in identifying these signaling pathways. Such biochemical events include cytoplasmic alkalinization through activation of the Na^+-H^+ antiporter, changes in the intracellular calcium ion concentration which are specific for integrin, ligand, and cell type although the exact mechanism is unclear, and inositol lipid metabolism[34].

An additional mechanism triggered by integrin-mediated signaling is tyrosine kinase activity. Protein tyrosine phosphorylation is an early event detected in response to integrin stimulation. Several studies have demonstrated altered tyrosine phosphorylation patterns in many cell types. Clustering of integrin $\alpha_3\beta_1$ on

KB human epidermal carcinoma cells induces an enhanced transient tyrosine phosphorylation of a complex of proteins of 115-130 kDa[35]. Also, adhesion and spreading of mouse NIH 3T3 fibroblast cells on FN or anti-integrin antibodies leads to rapid tyrosine phosphorylation of a range of proteins of similar molecular weight[36]. Aggregation of human platelets and engagement of integrin $\alpha_{IIb}\beta_3$ by fibrinogen induces tyrosine phosphorylation of a 125 kDa protein identified as the protein tyrosine kinase, pp125^{FAK}[37]. It has been demonstrated that upon integrin ligation the major tyrosine phosphoprotein in the carcinoma and fibroblast cells that becomes phosphorylated is also pp125^{FAK}[38,39].

pp125^{FAK} - Focal Adhesion Kinase

pp125^{FAK} was the first discovered member of a new family of non-receptor tyrosine kinases[40,41]. It was initially characterized as a major phosphotyrosine-containing protein of 125 kDa in pp60$^{v\text{-}src}$ transformed chicken embryo cells[42]. Isolation of the cDNA and amino acid sequencing revealed that pp125 was a cytoplasmic protein tyrosine kinase, and immunofluorescent studies localized it to focal adhesions, hence the designation focal adhesion kinase[43]. Later, a focal adhesion protein tyrosine kinase was characterized in mouse which was phosphorylated in response to cell attachment to FN[44].

FAK is a unique tyrosine kinase in that its catalytic domain is centrally located in the molecule and flanked by amino and carboxy terminal domains of approximately 400 amino acids each. The amino terminus of FAK contains a putative integrin-binding site. An *in vitro* translated fragment of the amino terminus corresponding to amino acid residues 31 to 376 can effectively bind synthetic

peptides of β integrin cytoplasmic domains[45]. The carboxy terminal 150 amino acids contain sequences which target FAK to focal adhesions[46]. This sequence is called a focal adhesion targeting, or FAT, sequence. Overlapping the FAT sequence in the carboxyl terminus is a binding site for the cytoskeletal protein paxillin[47]. At position 397 of FAK there is a tyrosine residue which is the autophosphorylation site of FAK[48]. Concomitant with phosphorylation at Tyr397 is activation of FAK[37]. A distinct feature of FAK that distinguishes it from other kinases, such as the src family of kinases, is that it lacks src homology (SH) 2 and SH3 domains. SH2 and SH3 domains mediate protein-protein interactions[49]. SH2 domains bind to phosphotyrosine residues, and SH3 domains bind to proline-rich sequences on proteins. However, although FAK does not contain SH2 or SH3 domains, phosphorylated Tyr397 of FAK has been demonstrated to serve as a binding site for the SH2 domain of src and fyn in fibroblasts[50].

FAK is a widely expressed protein found in many tissues and cell lines[41], including some lymphocyte cell populations[51]. cDNAs have been described in several species, and comparison of the amino acid sequences deduced from the cDNAs has revealed a high sequence similarity and 91% identity[41]. A FAK-related nonkinase (pp41/43^{FRNK}) has been isolated that encodes only the carboxy terminal domain of FAK[52]. A proposed function for FRNK employs it as an endogenous regulator of FAK by inhibiting FAK phosphorylation and formation of focal adhesions[53]. Recently, there have been kinases discovered to be additional members of the FAK family of non-receptor tyrosine kinases. FakB is a tyrosine kinase antigenically related to pp125^{FAK} that is phosphorylated and activated upon antigen receptor cross-linking with antibody in human T and B lymphocytes[54]. From rat and human, a 113 kDa cell adhesion kinase β (CAKβ) was identified with

homology to FAK that is localized to areas of cell-cell attachment[55]. Proline-rich tyrosine kinase 2 (PYK2) was isolated from a human brain cDNA library, and appears to be a neuronal member of the FAK family[56]. A novel related adhesion focal tyrosine kinase (RAFTK), which contains the same structural features as FAK, was identified and characterized from human megakaryocytes and brain[57].

FAK can also be phosphorylated by other stimuli besides integrins. Growth factors, hormones, neuropeptides, lysophosphatidic acid, and aggregation of IgE receptors on rat basophilic leukemia cells each can induce FAK phosphorylation[58,59,60,61]. It is unclear whether the function of FAK under these stimuli serves the same purpose as integrin stimulation or whether it has a distinct function.

Signaling in Focal Adhesions

Integrin receptors have short cytoplasmic domains with no intrinsic enzymatic activity. The nature of their function as signal transducers became the question, as it was discovered that integrins could mediate activation of signaling pathways. This phenomenon is not without precedent, though. There are a number of receptors with short cytoplasmic domains that interact with members of the src family of kinases which are anchored to the membrane with lipid anchors, and it is through activation of the kinases that signal transduction is mediated. For example, CD4 and CD8 on lymphocytes interact with lck, TCR in T cells interacts with fyn, BCR in B cells associates with lyn, and the immunoglobulin E receptor in mast cells and basophils coimmunoprecipitates with src family members[30].

Current models of integrin-mediated signaling postulate that intermediary signaling molecules are associated with or recruited to integrin cytoplasmic tails, and it is these molecules which initiate the cascade of signaling events into the cell after ligand binding by the integrin receptors[31]. In many cell types, it has been demonstrated that integrin ligation by binding to specific ECM proteins or by clustering with anti-integrin antibodies and oncogenic transformation results in enhanced tyrosine phosphorylation of proteins of 115-130 kDa, with one of the major phosphoproteins identified in this cluster as pp125^{FAK}[37,38,39,44,62]. The early increase in phosphorylation suggests the involvement of a protein tyrosine kinase. As FAK has been shown to bind to synthetic peptides of β integrin membrane-proximal cytoplasmic domains[45] and contains a FAT sequence[46], it is a likely candidate for the initial sequences of the signaling cascade. It has been hypothesized that integrin clustering may induce a conformational change in FAK which allows it to autophosphorylate or become susceptible to phosphorylation by other kinases, such as the src family of kinases. Integrin clustering may also allow the close juxtaposition of FAK molecules so that transphosphorylation can occur and allow for activation of the tyrosine kinase activity of FAK[41].

Integrin ligation also recruits many cytoskeletal proteins to the focal adhesions. The cytoskeleton is a complex network of protein filaments in the cytoplasm and is important in regulating cell shape. Treatment of platelets with cytochalasin D, a drug which disrupts the actin cytoskeleton, inhibits tyrosine phosphorylation of FAK, indicating an intact cytoskeleton is required for focal adhesion signaling[37]. The reorganization of cytoskeletal proteins changes cell shape which is necessary in a mobile cell. Some of the cytoskeletal proteins, such as paxillin, are regulated by tyrosine phosphorylation and are tyrosine

phosphorylated in focal adhesions. Paxillin can bind to the carboxyl terminus of FAK[47] and may be a substrate for FAK[62]. Phosphorylation of cytoskeletal proteins may regulate the formation of focal adhesions and recruitment of proteins containing SH2 and SH3 domains. For example, tyrosine phosphorylated paxillin may complex with the SH2 domain of tensin, an actin-binding, focal adhesion protein[62]. Through its SH3 domain, src can bind to the proline-rich region of paxillin[63] and through its SH2 domain is capable of binding to tyrosine phosphorylated FAK[48]. In these conformations src may phosphorylate substrates. The association of src and FAK may also serve to prolong the activity of both FAK and src by restricting access of phosphatases which may dephosphorylate and down-regulate the activity of FAK. FAK, in turn, may prevent the negative regulatory site of src from interacting with itself. Csk and syk kinases also localize to focal adhesions and may participate in the signaling cascade.

Beyond the focal adhesion, signaling progresses into the cell and to the nucleus by integration into some already well-characterized pathways. SH2 and SH3 adaptor proteins are composed exclusively of SH2 and SH3 domains and mediate important protein-protein interactions[64]. Growth factor receptor-bound protein 2 (Grb2) is such an adaptor protein that links receptor tyrosine kinases to the Ras pathway via association with Sos, which is a guanine nucleotide exchange factor and activator of Ras. Adhesion of murine fibroblasts to FN promotes association of the SH2 domain of Grb2 to the tyrosine phosphorylated residue 925 of FAK[65]. Downstream of Ras, mitogen-activated protein (MAP) kinases become activated. Integrin engagement of murine and rat fibroblasts can also activate MAP kinases and facilitate their translocation from the cytosol to the nucleus[66]. As MAP

kinase can phosphorylate and activate transcription factors, integrin stimulation may thus regulate gene expression.

The Ras, MAP kinase, and FAK pathways, as well as other pathways[31], which are regulated by integrins are also regulated by other growth factor receptors. As cells are usually bombarded by a variety of stimuli, integrins and other receptors may cross-talk between pathways and synergize their signals to enhance or suppress effects. Rho and rac, two members of the ras-related superfamily of low molecular weight GTP-binding proteins, are important regulators of actin polymerization and, thus, formation of focal adhesions and membrane ruffling, respectively[67,68]. Rho and rac mediate integration of signals from growth factor receptors and integrins. This allows for a complex network of regulatory systems to control focal adhesion formation, migration, differentiation, and proliferation.

Integrins on T Lymphocytes

T lymphocytes have been found to express a variety of integrins. The β_1 integrin family, in particular, is well represented. On resting T cells, there is abundant expression of $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$, but they are only minimally adhesive to their ligands, fibronectin for $\alpha_4\beta_1$ and $\alpha_5\beta_1$ and laminin for $\alpha_6\beta_1$. There is a lesser amount of $\alpha_3\beta_1$ and only traces of $\alpha_1\beta_1$ and $\alpha_2\beta_1$, although populations with heterogeneous integrin expression may exist[18]. After activation of $CD4^+$ T cells through the TCR, there is enhanced binding activity of $\alpha_4\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ detectable after ten minutes without change in the level of expression of the integrins[24]. Signaling through the TCR is necessary as a murine T cell clone

lacking a TCR fails to show increased adhesion to ECM proteins in response to antigen stimulation by antigen presenting cells[69]. The β_1 integrins are also referred to as the VLA proteins for "very late activation" antigens, reflecting their elevated binding activity only after activation. VLA-4 and VLA-5 represent $\alpha_4\beta_1$ and $\alpha_5\beta_1$, respectively, and the other β_1 integrins are named in a similar fashion. Memory T cells express three- to four-fold more β_1 integrins and exhibit more efficient binding to FN and laminin than naive T cells[70].

A β_2 integrin family member is also found on T cells. $\alpha_L\beta_2$ or LFA-1 is expressed on T cells. After engagement of the TCR, there is activation of LFA-1 to a high affinity binding state for its ligand, ICAM-1. This interaction strengthens conjugate formation between a T cell and an APC or a target cell[71].

T cells have also been found to express β_3 integrins. 25% of freshly isolated human peripheral $CD3^+$ T cells express $\alpha_v\beta_3$[72]. A panel of murine dendritic epidermal T cell lines and hybridomas that are TCR γ/δ^+, as well as Concanavalin A-stimulated splenic T blasts but not resting spleen, lymph node, or thymus cells react with an antibody specific for the VNR[28,73]. These cells bind to fibronectin, fibrinogen, and vitronectin, but not to the control protein ovalbumin, and binding is inhibitable by the tetrapeptide RGDS.

Integrins as Accessory Molecules in T Cell Activation

Several integrins have been found to be expressed on T lymphocytes[18]. These receptors for ECM proteins have been studied to determine their functional role in immune responses. Most of the studies have been performed with $CD4^+$ T

cells and look at the contribution of β_1 integrins or VLA proteins in T cell activation. Matsuyama et al[74] demonstrated that FN interacted with VLA-5 ($\alpha_5\beta_1$) on purified CD4+ T cells isolated from human peripheral blood and synergized with anti-CD3 antibody to induce proliferation. Neither FN nor anti-CD3 alone could induce proliferation, but collagen and not laminin co-immobilized with anti-CD3 antibody could moderately promote T cell proliferation. Anti-VLA-5 antibody and RGDS peptide could effectively inhibit proliferation stimulated by FN and anti-CD3. Shimizu et al[75] also showed that FN and anti-CD3 could stimulate proliferation of resting CD4+ T cells from human peripheral blood lymphocytes (PBL). They found that VLA-5 and VLA-4 mediated the costimulation of FN, but RGD peptide was only capable of blocking binding of VLA-5 and VLA-4 to FN and not costimulation, whereas CS-1 peptide from the alternatively spliced IIICS domain of FN that is bound by VLA-4 could block proliferation. In addition, they found that laminin was bound by VLA-6 and also stimulated anti-CD3-induced proliferation, but ECM proteins fibrinogen, collagen, and VN had no effect. As confirmation of the FN receptors mediating the anti-CD3-induced proliferation of CD4+ T cells, Nojima et al[76] demonstrated that VLA-4 could also mediate costimulation of T cell activation in an RGD-independent manner by binding the CS-1 domain of FN.

To address the mechanism of integrin-mediated, anti-CD3-induced proliferation, Davis et al[70] showed that FN not only enhanced binding of the T cells to the anti-CD3 coated surface to allow greater association of anti-CD3 with the TCR/CD3 complex and more effective signaling, but that FN delivered a costimulatory signal that synergized with anti-CD3 signaling. Immobilized FN introduced to the T cells on a separate surface as anti-CD3 was able to function as a costimulatory molecule. O'Rourke and Mescher[77] demonstrated activated

adhesion and enhanced phosphoinositide hydrolysis of murine CTL clones when the cells were pretreated by cross-linking the TCR and then incubating on FN.

It is important to identify the biochemical events triggered by the integrin-mediated costimulation of T cell activation and proliferation. Nojima et al[78] reported that engagement of $\alpha_4\beta_1$ by cross-linking with a β_1 monoclonal antibody (mAb) induced rapid tyrosine phosphorylation of a 105 kDa protein (pp105) in the human T lymphoblastic cell line H9 and in resting peripheral T cells. Tyrosine phosphorylation of cellular proteins is an early and obligatory event in T cell activation, suggesting that activation of tyrosine kinase activity upon integrin engagement plays a role in the transduction of integrin-mediated costimulation. Nojima et al[78] did not identify pp105, but Maguire et al[79] were able to identify a 116 kDa protein in Jurkat T cells and human T cell blasts that was tyrosine phosphorylated after triggering the TCR/CD3 complex or binding to FN as $pp125^{FAK}$. Co-immobilization of anti-CD3 antibody and FN synergistically increased the phosphorylation of FAK. The integrins that mediated the signals to induce tyrosine phosphorylation of FAK were $\alpha_4\beta_1$ and $\alpha_5\beta_1$ binding to FN, as well as $\alpha_4\beta_1$ binding to VCAM-1. Nojima et al[80] subsequently demonstrated the tyrosine phosphorylation of pp120, in addition to pp105, in the H9 cell line upon engagement of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ with FN and $\alpha_4\beta_1$ with VCAM-1 and identified pp120 as $pp125^{FAK}$. FAK has been implicated in the integrin-mediated signaling events of many non-lymphoid cells as well[37,38,39,44,62], and its identification in human T cells suggests that FAK may be involved in integrin-mediated costimulation of T cell activation and proliferation.

However, Kanner et al[54] did not find enhanced tyrosine phosphorylation of pp125^{FAK} after engagement of the antigen receptors of T and B cells, but found that a putative FAK homologue, fakB, showed augmented phosphorylation. FakB formed complexes with ZAP-70 even before antigen stimulation and may have a specialized role in T cell signaling pathways.

There have been a few reports that studied the β_3 integrin VNR on T cells. In murine γ/δ^+ dendritic epidermal T cell lines, the VNR binding to FN, VN, and fibrinogen in an RGD-dependent manner was a necessary accessory molecule to costimulate cytokine production[81]. Also, binding of the VNR to ECM proteins in the presence of a chimeric protein of ζ-chain cytoplasmic domain and CD8 transmembrane and extracellular domain was sufficient to induce cytokine secretion in a TCR-deficient T cell hybridoma without antigen recognition[82]. Tyrosine phosphorylation of a 115 kDa protein immunologically distinct from FAK was stimulated by engagement of the VNR in the TCR-deficient T cell hybridoma and a γ/δ^+ TCR T cell hybridoma[83]. The VNR on these T cells is composed of a 140 kDa α subunit and a 95 kDa β subunit under non-reducing conditions, and under reducing conditions the α chain is made up of disulphide-linked 116 kDa and 23 kDa chains and the β chain is 98 kDa[73].

Takahashi et al[84] used a mAb to characterize an extracellular matrix receptor with an α chain of 140 kDa and a β chain of 95 kDa on lymphokine-activated killer cells. Spleen cells activated with IL-2 or Con A expressed reactivity to the mAb after four days of stimulation and increased the expression for at least fourteen days after stimulation. The mAb blocked binding of the killer cells to FN, VN, and fibrinogen, but not laminin or collagen, and also inhibited cytotoxicity

towards target cells. In a murine CTL clone and a murine T_H hybridoma, adherence to FN, VN, and fibrinogen was blocked by the same mAb[85]. FN, VN, immobilized mAb, and to a lesser extent, fibrinogen synergistically enhanced anti-CD3-stimulated serine esterase release of the CTL clone and cytokine production of the T_H hybridoma. FN, VN, and immobilized mAb could independently trigger serine esterase release and cytokine production suggesting these stimuli could trigger antigen-dependent T cell functions. These effects could be efficiently inhibited by preincubation with mAb. Also using murine CTL clones, Ybarrondo et al[86] demonstrated that FN facilitated degranulation only when stimulation with soluble anti-TCR was cross-linked with a second antibody.

Purpose of this Study

In this study, we used murine CTL clones to ask if ECM proteins could be costimulatory in their activation. Several studies have been done with T_H cells and characterized the role of β_1 integrins in activation, but less has been studied in CTLs. We asked which ECM proteins had a costimulatory role and were capable of signal transduction. Next, we identified which integrin receptor(s) mediated the binding to the ECM proteins. We examined early signaling events in order to investigate the capacity in which integrin receptors acted in costimulation. As well, we attempted to identify the signaling pathways activated by integrin stimulation by studying proteins affected in CTL activation through ECM stimulation. From the conclusions drawn in this study, we discuss the physiological relevance of ECM costimulation in CTL activation.

CHAPTER II.

MATERIALS AND METHODS

Cells

Murine $CD8^+$ H-$2K^b$-specific cytotoxic T cell clone 11 was derived from peritoneal exudate cells of (B10.BR x B10.D2)F_1 mice 10 to 13 days after priming by intraperitoneal injection with 2 x 10^7 EL4, T lymphoma cells[87]. The K^b-specific CTL clone AB.1[88] was derived from limiting dilution culture from a line originally started as a BALB/c anti-C57BL/6 (H-2^d anti-H-2^b) mixed lymphocyte culture. The H-$2D^b$-specific clone 3/4 and D^d-allo-specific clone 10/1 were derived from C57BL/6 spleen cells[89,90].

The CTL clones were carried in RPMI 1640 (Life Technologies, Burlington, ON) supplemented with 10% FCS, 20 mM HEPES, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 100 µg/ml penicillin-streptomycin, and 2-mercaptoethanol (5 x 10^{-5} M) and maintained by weekly stimulation with irradiated (2500 rads) spleen cells from C57BL/6 (H-2^b) or BALB/c (H-2^d) mice (Jackson Labs, Bar Harbor, ME) and 12 U/ml human r-IL2. Experiments were performed 4-6 days after stimulation.

C37 and C50 are ova/I-A^d-specific $CD4^+$ T_H clones (provided by A.M. O'Rourke, Research Institute at Scripps Clinic, La Jolla, CA). The cells were stimulated every 14 days with BALB/c spleen cells along the same procedure as the CTL clones. The lck-deficient CTL clone, CTLL-2.lck^-, was provided by R.T.

Abraham (Mayo Clinic, Rochester, MN)[91]. The H-2^d-specific, fyn-deficient CTL clones, KO8 and 1E8[92], were generated from $fyn^{(-/-)}$ mice provided by R. Perlmutter and M. Appleby (Howard Hughes Medical Institute, Seattle, WA)

Con A blasts were derived from spleen cells resuspended at 10^6/ml and 10 ml cultured with 10 μg/ml of Con A in DMEM (Life Technologies, Burlington, ON) supplemented with 10% FCS and 1% penicillin-streptomycin. On day 4, the cells were split and expanded with IL-2.

Antibodies

The hybridomas producing mAb 145-2C11 (anti-CD3ε, obtained from ATCC), H57-597 (anti-TCRβ, obtained from ATCC), and PY-72 (anti-phosphotyrosine, obtained from Dr. B. Sefton) were grown in Protein Free Hybridoma Medium-II (Life Technologies, Burlington, ON) and the mAb purified by ammonium sulfate precipitation followed by either protein A or protein G chromatography, if required. Anti-CD51 (α_v), anti-CD49a (α_1), anti-CD49b (α_2), anti-CD49d (α_4), anti-CD49e (α_5), anti-CD49f (α_6), and anti-CD29 (β_1) mAb were purchased from PharMingen (San Diego, CA). We have two different mAb specific for CD61 (β_3), a hamster IgG purchased from PharMingen (San Diego, CA) and a mouse IgM purchased from Transduction Laboratories (Lexington, KY). Anti-$pp125^{FAK}$ antisera were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology Incorporated (Lake Placid, NY). The mAb specific for p120 was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY), and anti-vinculin was obtained from Sigma Immunochemicals (St. Louis, MO). Antibody to c-cbl was provided by A. Veillette (McGill University, Montreal, PQ).

Protein Immobilization

Antibodies were diluted to the concentrations of 0.1 to 20 µg/ml in PBS and 40 µl of the diluted antibodies were incubated in each well of 96-well Nunclon plates (Life Technologies, Burlington, ON) for 2 hours at 37°C. Co-immobilization with cellular FN (Upstate Biotechnology, Inc., Lake Placid, NY) or other ECM proteins at a final concentration of 10 µg/ml was carried out simultaneously in a total volume of 40 µl. Alternatively, for immunoprecipitations, protein immobilization was done in 60 x 15 mm plastic petri dishes (Fisher Scientific, Nepean, ON) in a volume of 5 ml. The wells or dishes were washed twice with PBS and blocked for 1.5 hours with 2% (w/v) BSA in PBS at 37°C. The wells or dishes were once again washed twice with PBS before use in the assays.

CTL Degranulation Assay

Degranulation was assayed by measuring the serine esterase activity released into the supernatant medium. 10^5 CTL clones in 150 µl RPMI containing 2% normal calf serum were added to microtitre wells immobilized with the appropriate proteins. Plates were incubated for 3 to 4 hours at 37°C, after which 20 µl aliquots of supernatant were assayed for serine esterase activity using 150 µl of a mixture of *N* -benzyloxycarbonyl-L-lysine thiobenzyl ester, hydrochloride (Calbiochem, La Jolla, CA) and 5'5-dithiobis(2-nitrobenzoic acid) (Sigma, St. Louis, MO) dissolved in PBS. The reaction was allowed to proceed for 15 to 20 minutes and the A420 was determined with a microplate reader (Molecular Devices, Menlo Park, CA). Experiments were performed in triplicate and error bars indicate the standard deviation.

Anti-Phosphotyrosine Immunoblotting

10^5 cells in a volume of 40 µl D-PBS (Life Technologies, Burlington, ON) were added to wells that had been coated with the indicated proteins. In the inhibition studies, cells were incubated with 10 µg/ml of antibody or the indicated amount of inhibitor for 15-20 minutes on ice prior to adding to the wells. Cells were allowed to settle without centrifugation and the incubation times indicated were initiated upon addition of the cells to the wells and include the time required for the cells to settle on the bottom of the wells. After incubation at 37°C, cells were immediately lysed by the addition of an equal volume of 2X Laemmli SDS-electrophoresis sample buffer and boiled. Total cellular protein (10^5 cell equivalents per lane) was loaded onto either 7.5% or 10% gels and subjected to electrophoresis. Proteins were transferred to Immobilon-P (Millipore Corp., Bedford, MA) in 20% methanol/20 mM Tris base/96 mM glycine for 4 to 5 hours at 150 mAmps. Immunoblotting was performed using PY-72 and developed with goat anti-mouse coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), followed by detection with Enhanced Chemiluminescence (Amersham, Buckinghamshire, England) or Renaissance (Dupont NEN Research Products, Boston, MA).

FACScan Analysis

10^6 cells were incubated with unlabelled first antibody for 30 minutes at 4°C in PBS supplemented with 0.5% FCS and 0.1% sodium azide (IF buffer). The cells were washed twice with IF buffer, then incubated with the appropriate FITC-conjugated second antibody (Jackson ImmunoResearch Laboratories, Inc., West

Grove, PA) for 30 minutes at 4°C. Analysis of formaldehyde fixed cells was performed on 5,000 cells per sample using a FACScan (Becton Dickinson, Mountain View, CA) with Lysis II software. Second antibody alone and unstained controls were included.

Surface Biotinylation and Immunoprecipitation

Cells resuspended at 10^7/ml in D-PBS were incubated for 10 minutes at room temperature with 25 µl of Sulfo-NHS-Biotin (Pierce, Rockford, IL), diluted in water at 4.2 mg/ml, for every 10^7 cells. The reaction was quenched by adding an equal volume of media containing 2% FCS and incubating on ice for 5 minutes. The cells were washed 3X with D-PBS, then lysed at 10^7/ml with 1% Nonidet P40 (NP40) (United States Biochemical, Cleveland, OH) in PBS for 25 minutes on ice. Postnuclear lysates were precleared with 10 µl normal rabbit serum and 10 µl packed protein A Sepharose beads (Pharmacia Biotech, Picataway, NJ) for every 10^7 cell equivalents by rotating for 1 hour at 4°C. The precleared lysate was immunoprecipitated by incubating 2 x 10^6 cell equivalent with 2 µg of primary antibody on ice for 15 minutes. After rabbit second antibody was incubated on ice for another 15 minutes, 40 µl of packed protein A beads were added and rotated for 1 hour at 4°C. The beads were washed 5 times with lysis buffer containing 1% deoxycholate and 0.1% SDS, resuspended in 60 µl 1X Laemmli sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reducing sample buffer, and boiled. After electrophoresis and transfer to Immobilon-P (Millipore Corp., Bedford, MA), the immunoprecipitated proteins were blotted with streptavidin coupled to horseradish peroxidase (Boehringer Mannheim Canada, Laval, PQ) and detected with Enhanced Chemiluminescence.

Immunoprecipitation from Stimulated Cells

Plastic petri dishes were coated with ligands as described above. 7.5×10^6 cells were incubated in the dishes for 30 minutes at 37°C and lysed by the addition of an equal volume of 1.5% NP40 in PBS containing 1 mM vanadate for 25 minutes. Postnuclear lysates were immunoprecipitated with 2-5 µg of antibody incubated for 15 minutes on ice followed by 40 µl of packed protein A beads rotated for 1 hour at 4°C. The beads were washed 5 times in 1% NP40 in PBS, resuspended in 60 µl 1X Laemmli SDS-PAGE reducing sample buffer, and boiled.

CHAPTER III.

RESULTS

Fibronectin is a Costimulator for T Cell Activation

Several studies have been done to indicate that FN is a costimulatory molecule for $CD4^+$ T cells[69,70,74,75,76]. We set out to determine if FN had any similar role in $CD8^+$ CTL. We first looked to see if FN could have any effect on the degranulation of our $CD8^+$ CTL clone 11. We co-immobilized various amounts of anti-CD3 mAb in the presence or absence of FN on wells of a microtitre plate and determined the amount of degranulation, an assay of CTL activation, as measured by serine esterase release from the CTL clones into the media supernatant. FN greatly enhanced the maximal amount of degranulation stimulated by anti-CD3 (145-2C11), particularly at sub-stimulatory concentrations of anti-CD3 where there was little degranulation (Figure 1A.). There was about a ten-fold reduction in the amount of antibody required to trigger degranulation when co-immobilized with FN (Figure 1B.). The enhancement by FN was seen at FN concentrations as low as 2.5 µg/ml which is equivalent to 100 ng/well and was maximal at 10 µg/ml (Figure 1A., inset). A wide range of FN concentrations alone were insufficient to trigger degranulation (Figure 1A., inset). Identical results were obtained with anti-TCR (H57-597) co-immobilized with FN (data not shown).

As FN could potentiate anti-CD3 stimulated degranulation, we next looked to see if FN could affect upstream signaling events by examining anti-TCR stimulated tyrosine phosphorylation of cellular proteins. Cells were stimulated with various

amounts of immobilized anti-TCR in the presence or absence of FN for 30 min. The cells were then lysed, the total cellular lysates were subjected to reducing SDS-PAGE, and the tyrosine phosphorylation of total cellular proteins was determined by anti-phosphotyrosine immunoblotting. We observed that FN synergistically enhanced tyrosine phosphorylation events induced by anti-TCR (Figure 2). Identical results were obtained with anti-CD3 (data not shown). Sub-stimulatory concentrations of anti-TCR that alone were insufficient to stimulate tyrosine phosphorylation showed significant phosphorylation of clusters of proteins in the molecular weight ranges of 115-125 kDa, 65-80 kDa, and 40 kDa when costimulated with FN. In the presence of FN, there was an identical pattern of tyrosine phosphorylated proteins with higher, stimulatory concentrations of anti-TCR, ie. 7.5 μg/ml, as with sub-stimulatory concentrations of anti-TCR, ie.0.5 μg/ml, suggesting that FN did not stimulate any new phosphorylation events.

The conditions that stimulated tyrosine phosphorylation events also stimulated serine esterase release, suggesting that the two responses may be functionally linked. Very similar results to those shown were observed with CTL clone AB.1 (Figure 7 and 9) with high correlation between the conditions required for the induction of tyrosine phosphorylation and serine esterase release.

Since the experiments from figure 2 were performed at only one time point, we did kinetic studies to determine the extent of FN-stimulated tyrosine phosphorylation events. As seen in figures 2 and 3, immobilized FN was able to independently induce tyrosine phosphorylation of a major protein of 116 kDa (pp116) in the group of proteins of 115-125 kDa. The transient phosphorylation of pp116 peaked at 20 minutes and decreased rapidly thereafter (Figure 3). Sub-

optimal concentrations of anti-CD3 alone could occasionally stimulate a very low level of phosphorylation of pp116, with figure 3 showing the maximum amount ever observed. When FN was co-immobilized with anti-CD3, the phosphorylation of pp116 was enhanced and potentiated beyond 40 minutes and included phosphorylation of the additional 65-80 kDa and 40 kDa protein clusters (Figure 3.

FN-Stimulated Responses are RGD-dependent

FN-facilitated responses can be inhibited by RGD peptides if the integrin receptor specifically recognizes the RGD sequence, eg. VLA-5. On the other hand, FN recognition can also be RGD-independent, as in VLA-4, which recognizes an entirely different sequence on a distinct region of FN. We used an RGD-containing peptide and a control RGE-containing peptide to determine if the FN-enhanced, anti-CD3-stimulated responses we observed were mediated by an RGD-dependent receptor. There was significant inhibition of serine esterase release and essentially complete inhibition of tyrosine phosphorylation of pp116 with GRGDSP but not GRGESP when cells incubated with the peptides were stimulated with FN and sub-stimulatory anti-CD3 (Figure 4). However, we were unable to inhibit the responses as effectively with GRDGNP (Figure 5), which is a weak inhibitor of cell attachment to VN[26]. These results suggest that VLA-4 is not mediating these events, and an RGD-dependent receptor(s) is responsible.

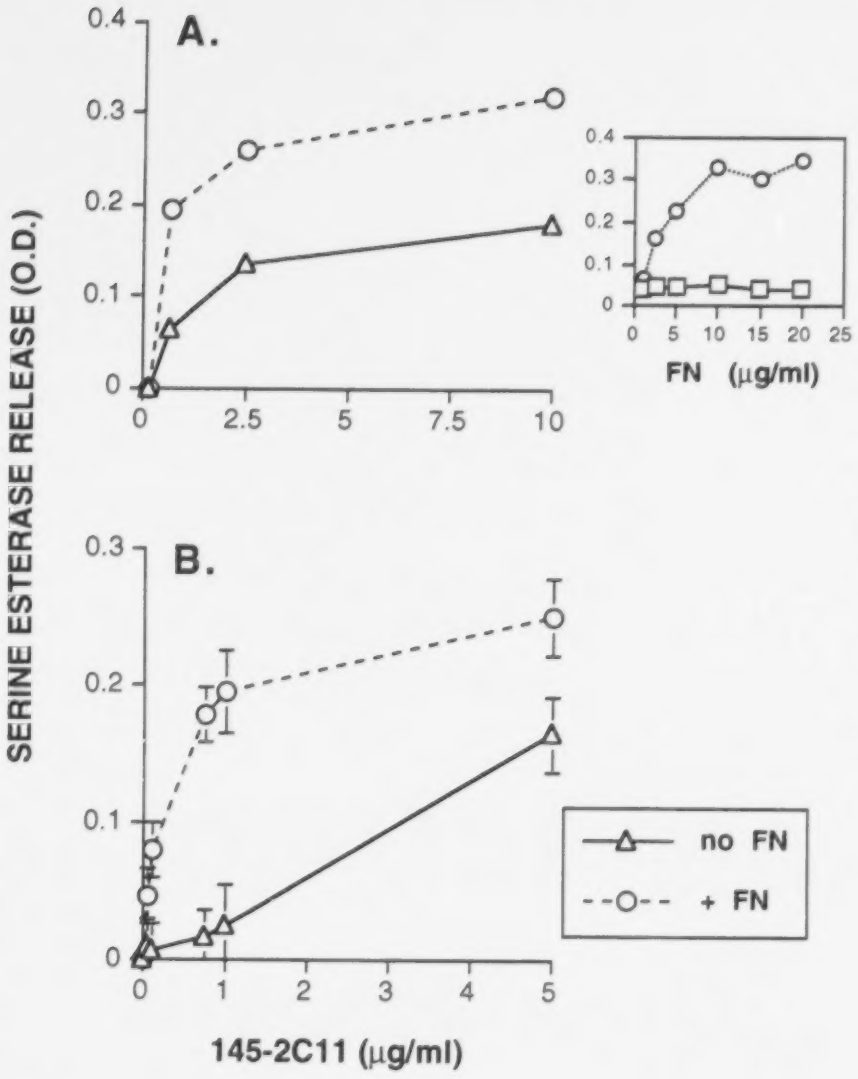

FIGURE 1. FN enhancement of anti-CD3 stimulated degranulation of CTL clone 11. Triplicate wells of a 96-well microtitre plate were coated with various concentrations of immobilized anti-CD3 (145-2C11) in the presence or absence of 10 μg/ml FN. 10^5 cells were added to each well and supernatants were assayed for serine esterase activity 4 hours later. A. and B. are two different experiments using different concentration ranges of antibody. The inset of A. is a titration of FN in the presence (circles) or absence (squares) of 1 μg/ml 2C11. Error bars indicate standard deviation, where visible.

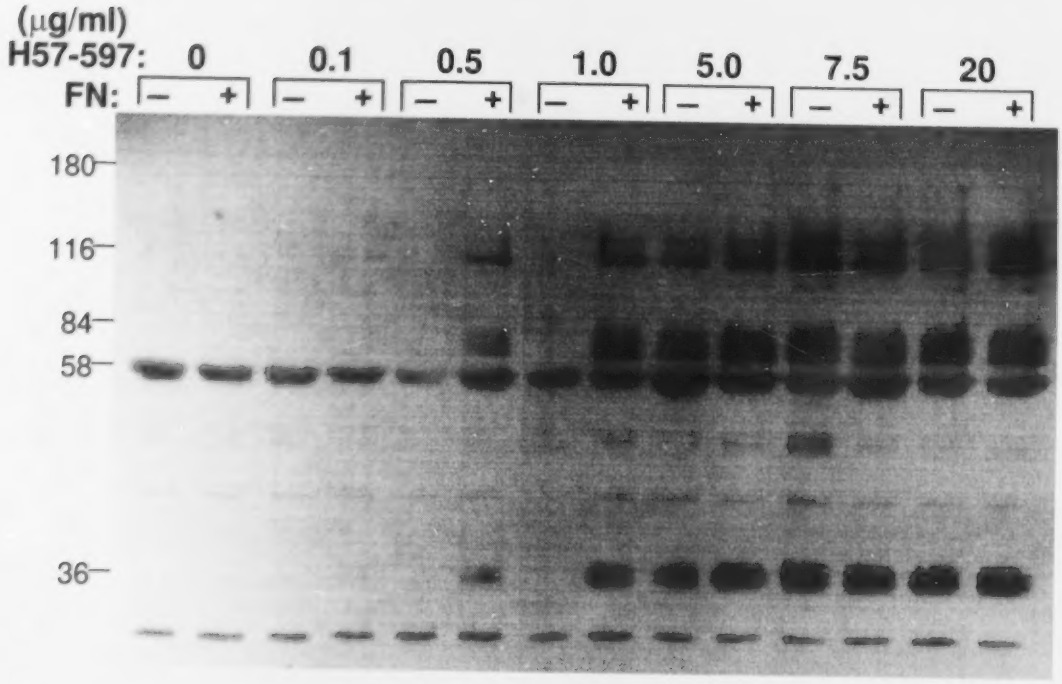

FIGURE 2. Synergistic enhancement of tyrosine phosphorylation events with FN and anti-TCR in CTL clone 11. 10^5 cells were added to each well of a microtitre plate coated with different concentrations of anti-TCR (H57-597) in the presence or absence of 10 μg/ml FN. After a 30 minute incubation, the cells were lysed and total cell lysates were subjected to SDS-PAGE and anti-phosphotyrosine immunoblotting. Molecular weight markers are indicated on the left of the blot.

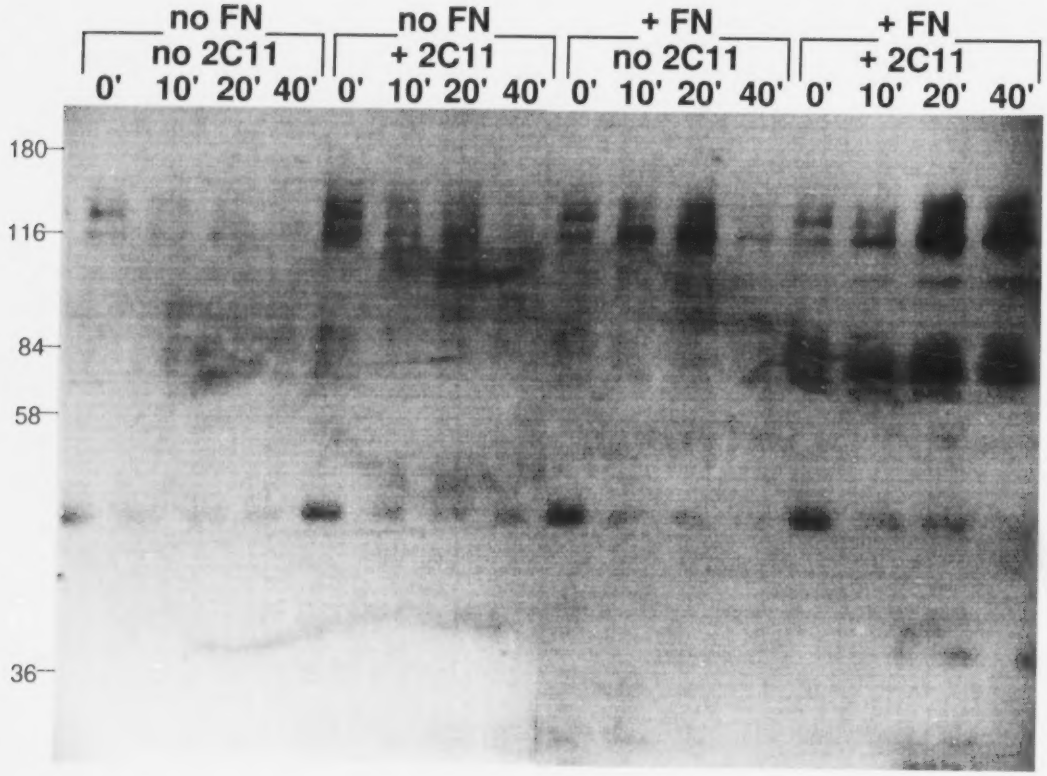

FIGURE 3. FN-stimulated tyrosine phosphorylation of a 116 kDa protein; enhanced and prolonged tyrosine phosphorylation events induced synergistically by FN and anti-CD3. Wells were coated with BSA (lanes 1-4), 1 μg/ml 145-2C11 (lanes 5-8), 10 μg/ml FN (lanes 9-12), or both 2C11 and FN (lanes 13-16). 10^5 clone 11 CTL were added to each well and incubated for various times, followed by anti-phosphotyrosine immunoblotting, as in Fig. 2.

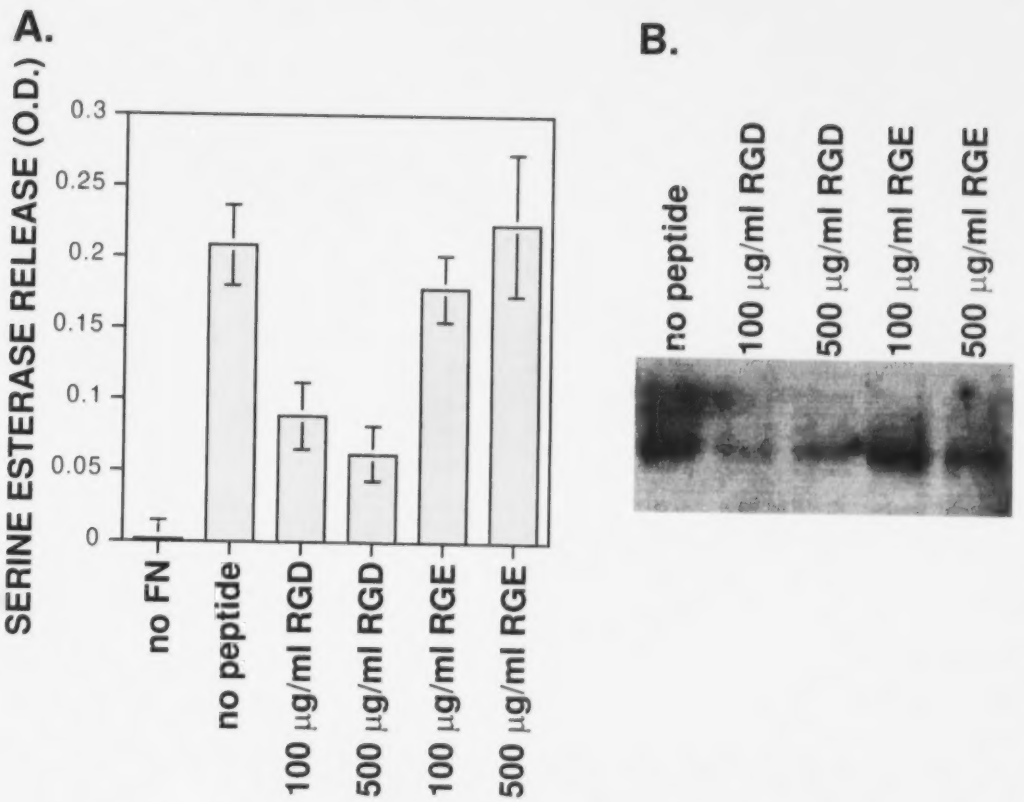

FIGURE 4. RGD inhibition of FN-enhanced degranulation and tyrosine phosphorylation of pp116 in CTL clone 11. GRGDSP or control GRGESP peptides were added to cells at a final concentration of 100 μg/ml or 500 μg/ml before incubation of 10^5 cells per well in wells co-immobilized with 1 μg/ml 145-2C11 and 10 μg/ml FN. A. Supernatants were assayed for serine esterase activity after a 4 hour incubation. B. Cells were incubated for 30 minutes and then assayed for phosphotyrosine content of pp116, as in Fig. 2.

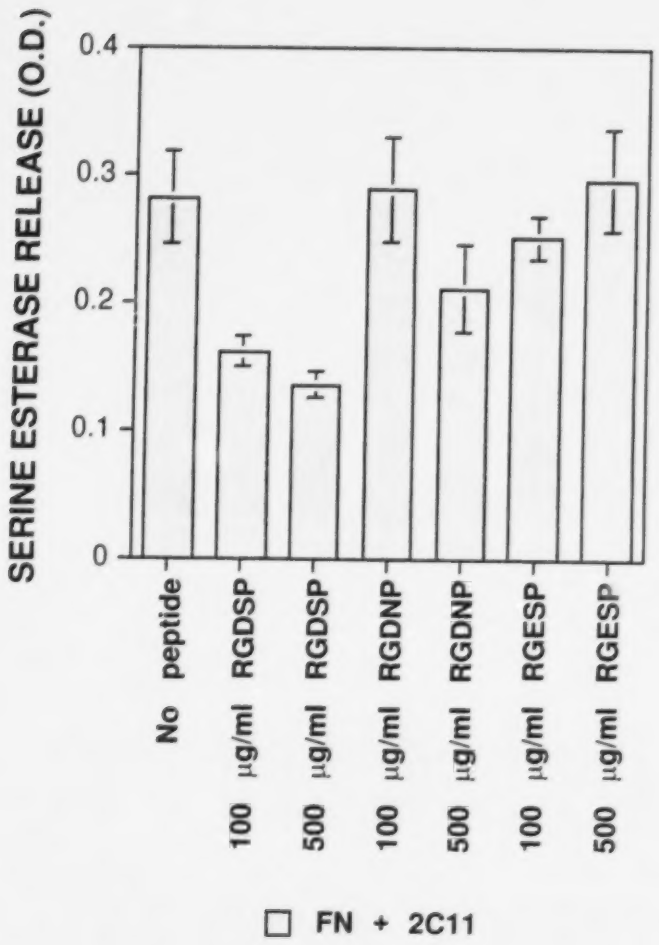

FIGURE 5. RGDSP, but not RGDNP, peptides inhibit degranulation of CTL clone 11 stimulated by FN co-immobilitzed with sub-stimulatory anti-CD3. GRGDSP, GRGDNP, or control GRGESP peptides were added to cells at a final concentration of 100 µg/ml or 500 µg/ml before incubation of 10^5 cells per well in wells co-immobilized with 10 µg/ml FN and 1 µg/ml 145-2C11. Supernatants were assayed for serine esterase release activity after a 4 hour incubation.

FN, VN, and FG can Stimulate T Cells

Since the responses we observed were dependent on the RGD sequence, we investigated whether any other ECM proteins, containing or lacking the RGD sequence, had any effects on CD3-mediated events. We co-immobilized FN, VN, fibrinogen (FG), collagen (types I and III), or laminin with a sub-stimulatory amount of anti-CD3 and measured degranulation by the CTL clone 11 and AB.1. FN, VN, and FG facilitated high levels of degranulation, whereas both types of collagen and laminin exhibited very little or no degranulation (Figures 6 and 7). These experiments were done at saturating concentrations for the ECM proteins, and over a wide range of collagen and laminin concentrations there was no significant change in the response (data not shown).

We next tested if the ECM proteins were able to stimulate the tyrosine phosphorylation in the CTL clones of the proteins in the 115-125 kDa range, since the phosphorylation of these proteins was inducible by FN. Similar to FN, VN and FG alone could induce the phosphorylation of the 115-125 kDa proteins, with pp116 as the prominent phosphoprotein (Figures 8 and 9). When FN, VN, and FG were co-immobilized with sub-optimal concentrations of anti-CD3, there was an enhancement of the phosphorylation of these proteins (Figures 8 and 9). Also, collagen types III and I and laminin, alone or with sub-stimulatory anti-CD3, were unable to stimulate phosphorylation of the 115-125 kDa proteins (Figures 8 and 9), consistent with the degranulation results. It is interesting to note that VN induced a higher level of phosphorylation than FN, but did not enhance degranulation to as great a degree as FN.

Two $CD4^+$ T_H clones (Figure 10), and Con A activated splenic T cell blasts (Figure 11) have also been examined for the ability of the ECM proteins to stimulate tyrosine phosphorylation. In all the clones tested, we found that FN and VN alone were able to induce the phosphorylation of the 115-125 kDa proteins. When co-immobilized with sub-stimulatory anti-CD3, the phosphorylation was enhanced in the clones and inducible in the Con A blasts. There was no phosphorylation seen with laminin, nor did it synergize with anti-CD3 to induce phosphorylation (Figures 10 and 11).

A β_3 Integrin is Expressed on T Cells

The specificity of the responses we observed suggested that a β_3 integrin may be expressed on the CTL clone 11 and utilized to mediate the responses. In particular, the vitronectin receptor (VNR) heterodimer, $\alpha_v\beta_3$, has the same specificity and recognizes the RGD sequence[17]. We investigated which of the known FN and VN receptors were expressed on the CTL clones by FACS analysis with the relatively few available antibodies to mouse integrins. As shown in figure 12, the CTL express CD3 and CD45, as predicted. There was abundant staining with CD61 (β_3) and significant staining with CD51 (α_v). CD29 (β_1) was detected on the CTL clones. We did not detect any expression of CD49d (α_4), in support that VLA-4 was not responsible for mediating the degranulation or tyrosine phosphorylation. However, there was CD49e (α_5) expression, suggesting the FNR is present on the CTL. We did not detect expression of CD49a (α_1), and there was significant expression of CD49b (α_2) and CD49f (α_6) subunits.

We have examined the integrin expression on two additional CTL clones and two T_H clones by FACS and observed positive staining for β_3 and detectable expression of α_v integrins on both CTL clones and one T_H clone (data not shown). Con A activated splenic T cell blasts also exhibited expression of β_3, consistent with previous studies[73].

To obtain biochemical evidence for the expression of β_3 integrin on the CTL clones, we used the same integrin antibodies as in the FACS analysis to perform immunoprecipitations from surface biotinylated cells and electrophoresed the samples on SDS-PAGE under reducing conditions. The IgG mAb to β_3 integrin immunoprecipitated two polypeptides consistent with the predicted molecular weights of 105 kDa for β_3 and 125 kDa for α_v (Figure 13). The mAb to α_v, α_4, and α_5 were ineffective in several immunoprecipitation experiments (Figure 13). Both the FACS analysis and immunoprecipitation data demonstrate that CTL do express a β_3 integrin, most likely coupled with α_v as the VNR.

β_3 Integrin Antibody can Stimulate Tyrosine Phosphorylation

Since the CTL express β_3 integrin, and possibly the VNR, we wanted to determine if a β_3 integrin was mediating the signal transduction events we observed when engaged by ECM proteins. The β_3 integrin antibody immobilized in microtitre wells was capable of inducing tyrosine phosphorylation of the 115-125 kDa bands in the CTL clone 11 (Figure 14A.). The kinetics of phosphorylation were similar to stimulation with VN (Figure 14B.) or FN (Figure 3) suggesting that the three stimuli triggered the same events. Immobilized α_5 and α_v antibodies were unable to stimulate phosphorylation (Figure 14A.). It is possible that these

antibodies do not cross-link the receptors in the appropriate way to induce phosphorylation. Even though the α_5 and β_1 antibodies were ineffective in this capacity does not imply that the FNR may mediate some events when the cells are incubated on FN.

Antibody inhibition studies were done to confirm that a β_3 integrin was mediating the stimulation of phosphorylation on VN (Figure 15A.) and FN (Figure 15B.). We used two different monoclonal antibodies to β_3 integrin which individually could partially block phosphorylation of pp116, in particular. The two antibodies in combination could completely inhibit the phosphorylation. However, a β_1 antibody and α_5 antibody did not have any effect, either alone or in combination. The β_1 antibody together with either of the β_3 antibodies only inhibited phosphorylation to the same extent as the β_3 integrin antibodies alone. Although the α_v antibody was ineffective at inhibition when used alone, there was almost complete inhibition when incubated with either β_3 integrin antibody. The β_1 antibody used in these experiments has been shown to recognize $\alpha_4\beta_1$. However, its ability to recognize β_1 in association with α_5 has not been characterized, and it is possible that if it does not recognize $\alpha_5\beta_1$ then it would not be able to block the interaction with FN. Regardless, these results demonstrate that a β_3 integrin can mediate the stimulation of tyrosine phosphorylation by FN and VN.

The β_3 integrin antibodies were used to try and inhibit degranulation of the CTL clones facilitated by FN and VN in response to sub-stimulatory anti-CD3. They did not significantly block degranulation (Figure 16), indicating that the antibodies were effective at blocking early signaling events, ie. phosphorylation, but not later events. It may be that since the cells were incubated for 4 hours before measuring

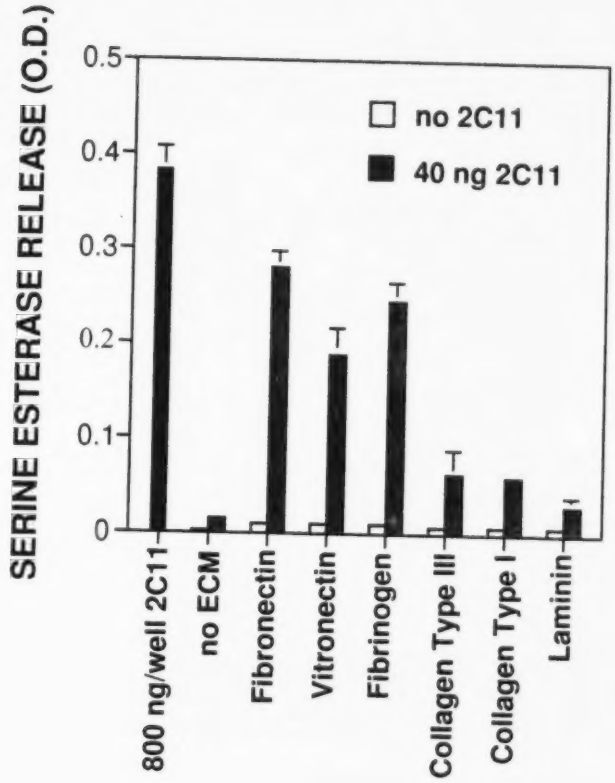

FIGURE 6. Anti-CD3 stimulated degranulation of CTL clone 11 facilitated by fibronectin, vitronectin, and fibrinogen. 0.4 μg (10 μg/ml) of ECM proteins were immobilized in the presence or absence of 40 ng (1μg/ml) 145-2C11 per well of a microtitre plate. 10^5 cells were added to each well and serine esterase activity was measured after a 4 hour incubation. Results were generated from triplicate wells, and error bars indicate standard deviation.

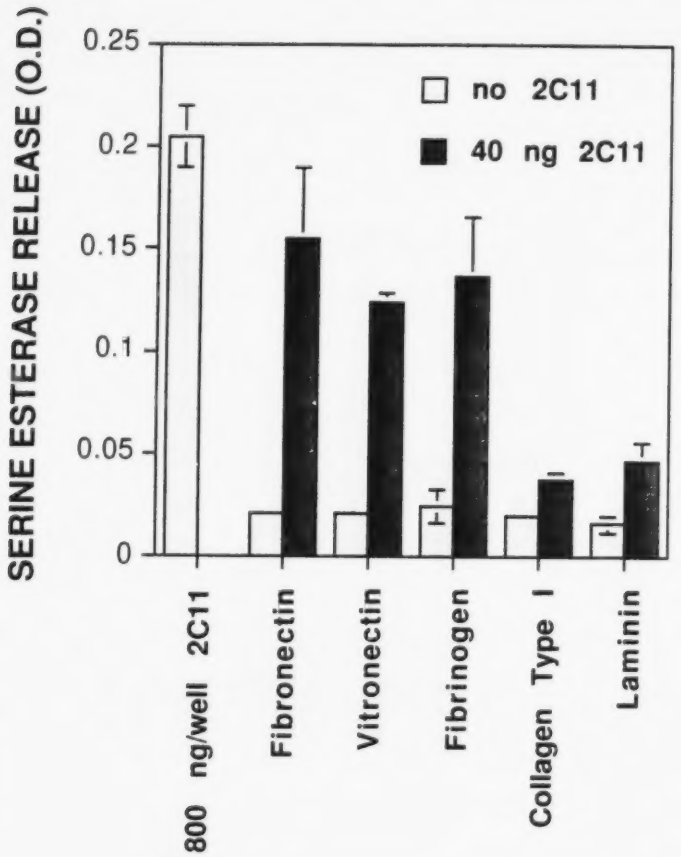

FIGURE 7. Anti-CD3 stimulated degranulation of CTL clone AB.1 facilitated by ECM proteins. Proteins were immobilized, as in Fig. 6. 10^5 cells were incubated in each well and assayed for serine esterase release, as in Fig. 6.

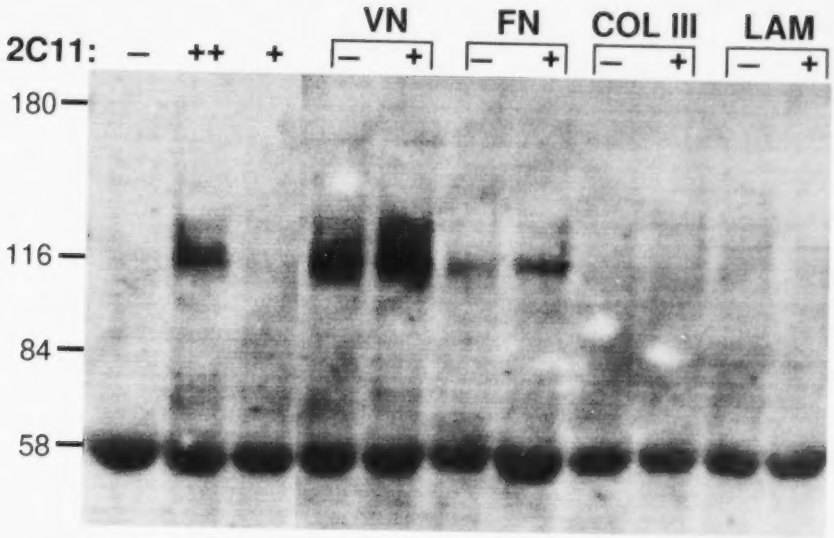

FIGURE 8. Tyrosine phosphorylation events in CTL clone 11 induced by ECM proteins in the presence and absence of anti-CD3. ECM proteins were immobilized in the presence or absence of 145-2C11, as in Fig. 6. 10^5 cells were incubated for 20 minutes in the wells, lysed, and total cell lysates were electrophoresed and immunoblotted with anti-phosphotyrosine. ++ indicates wells coated with maximally stimulatory (800 ng) 145-2C11. (LAM, laminin; COL III, collagen type III)

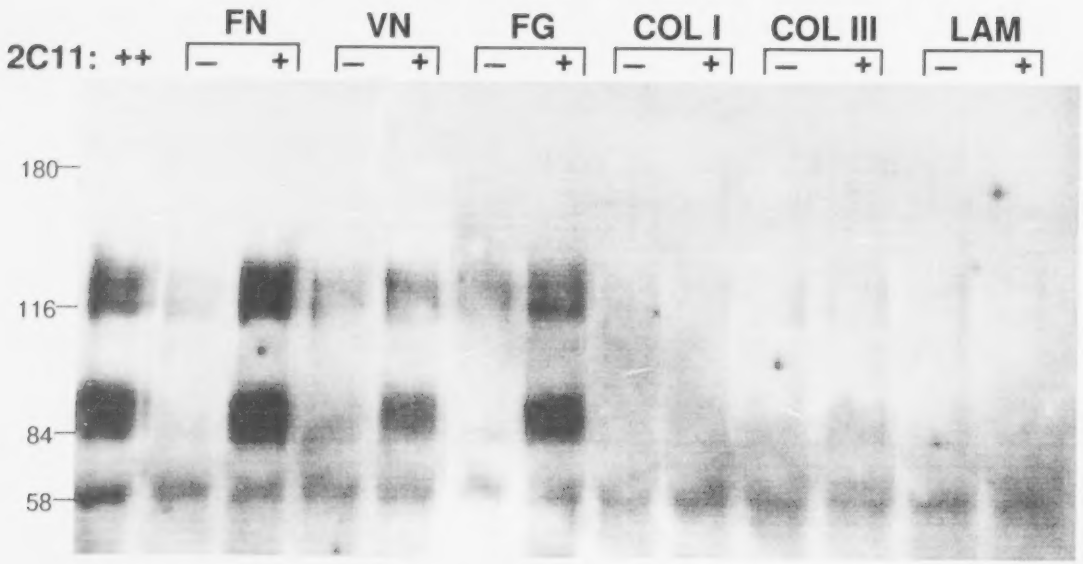

FIGURE 9. Tyrosine phosphorylation events in CTL clone AB.1 induced by ECM proteins in the presence and absence of anti-CD3. Microtitre plate wells were immobilized with ECM proteins, as in Fig. 6. Tyrosine phosphorylation of proteins were determined, as in Fig. 8. (COL I, collagen type I)

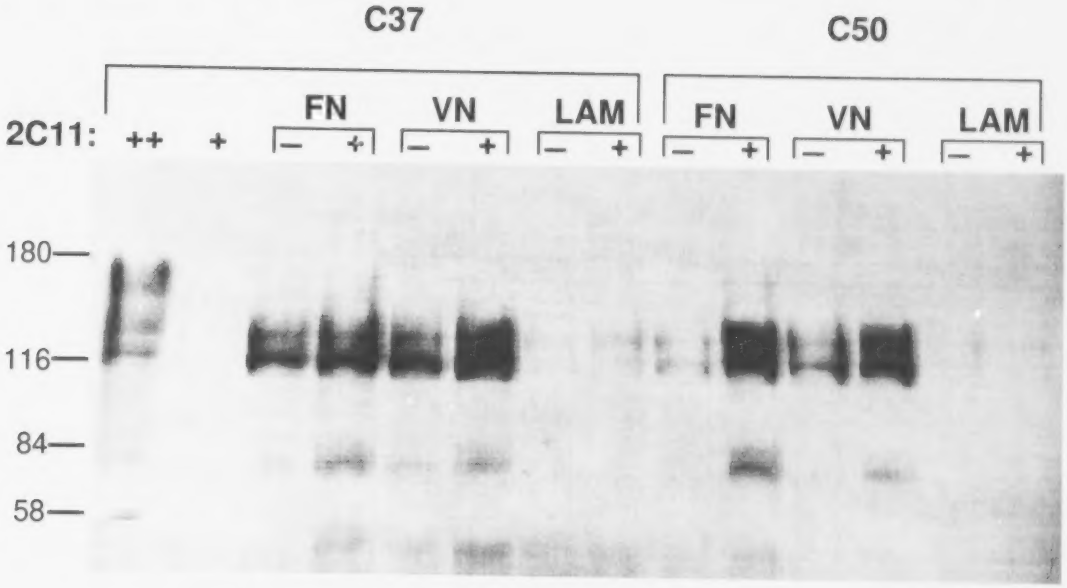

FIGURE 10. Tyrosine phosphorylation events induced by ECM proteins in the presence and absence of anti-CD3 in $CD4^+$ T_H clones. Microtitre wells were immobilized with ECM proteins, as in Fig. 6. Tyrosine phosphorylation of proteins from $CD4^+$ T_H clones, C37 and C50, were determined, as in Fig. 8.

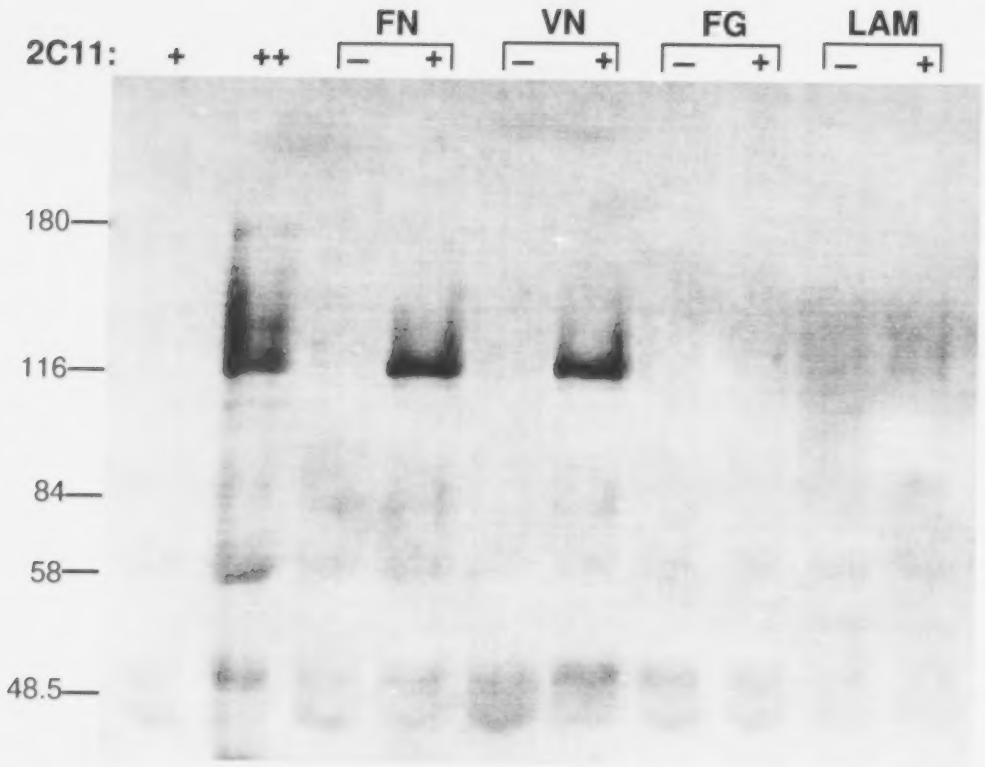

FIGURE 11. Tyrosine phosphorylation events in Con A activated splenic T cell blasts induced by ECM proteins in the presence and absence of anti-CD3. Microtitre plate wells were immobilized with ECM proteins, as in Fig. 6. 10^5 Con A activated T cell blasts per well were incubated for 20 minutes and prepared, as in Fig. 8.

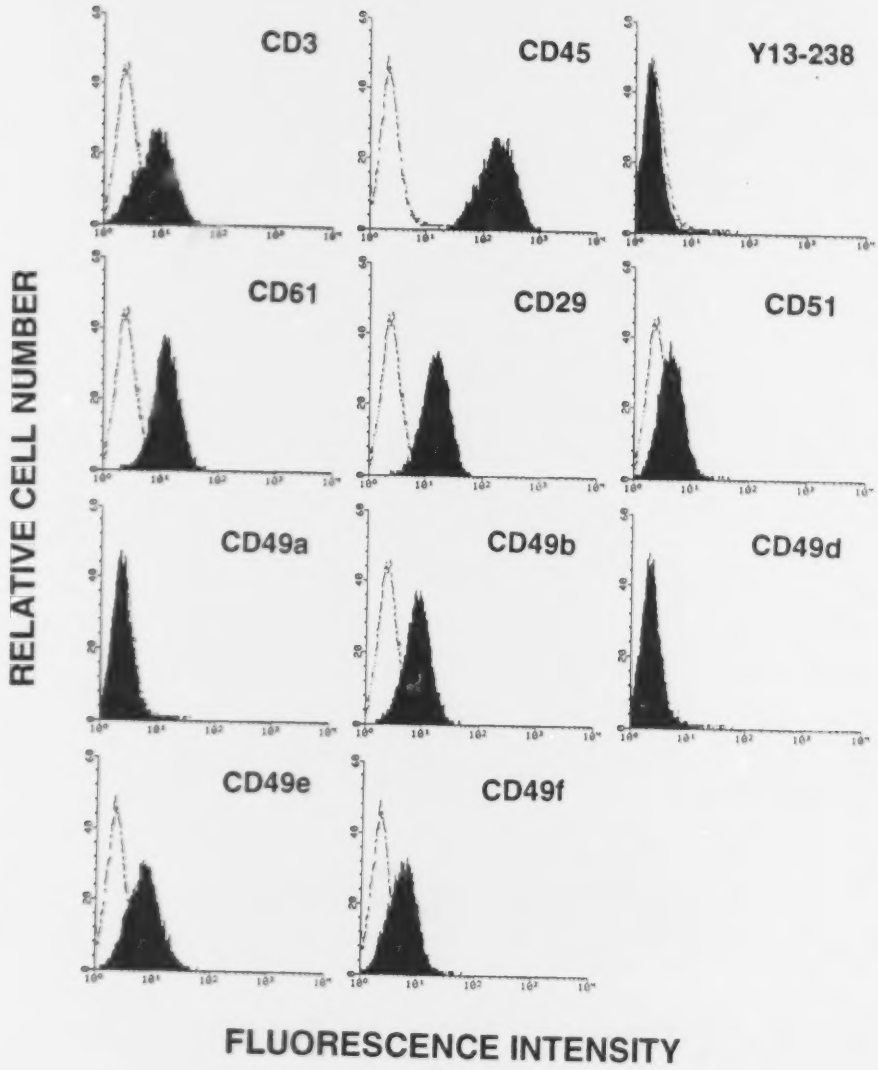

FIGURE 12. FACS analysis of the expression of integrins on CTL clone 11. 10^6 cells were incubated with the indicated antibody followed by the appropriate FITC-conjugated second antibody (solid profiles). CD61 was detected using the IgG mAb to β_3 integrin. The open profiles are of second antibody alone, and Y13-238 is an isotype control.

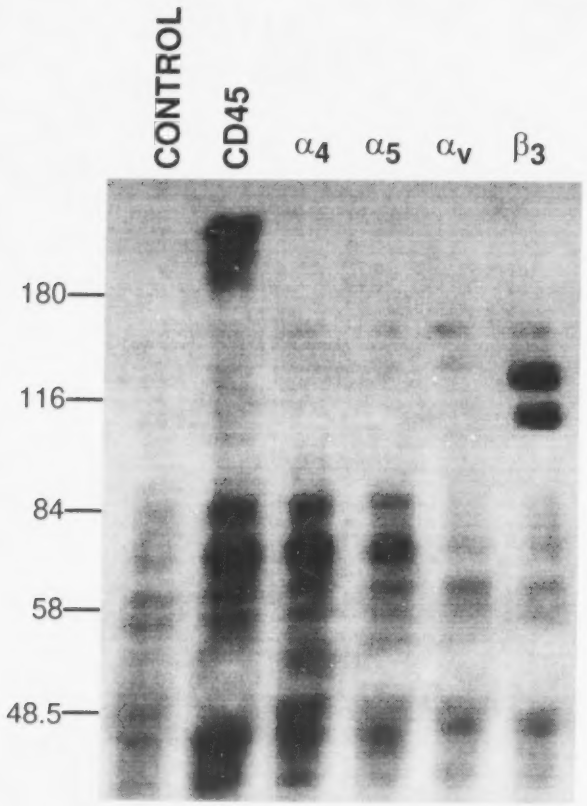

FIGURE 13. Immunoprecipitation of integrin subunits from surface biotinylated CTL clone 11. Cells were surface biotinylated, lysed in 1% NP40, and postnuclear lysates were precleared with normal rabbit serum. 2 x 10^6 cell equivalents were immunoprecipitated with the indicated mAb followed by the appropriate rabbit second antibody. Anti-β_3 is the IgG mAb. Immunoprecipitates were electrophoresed and blotted with streptavidin coupled to horseradish peroxidase and detected with Enhanced Chemiluminescence.

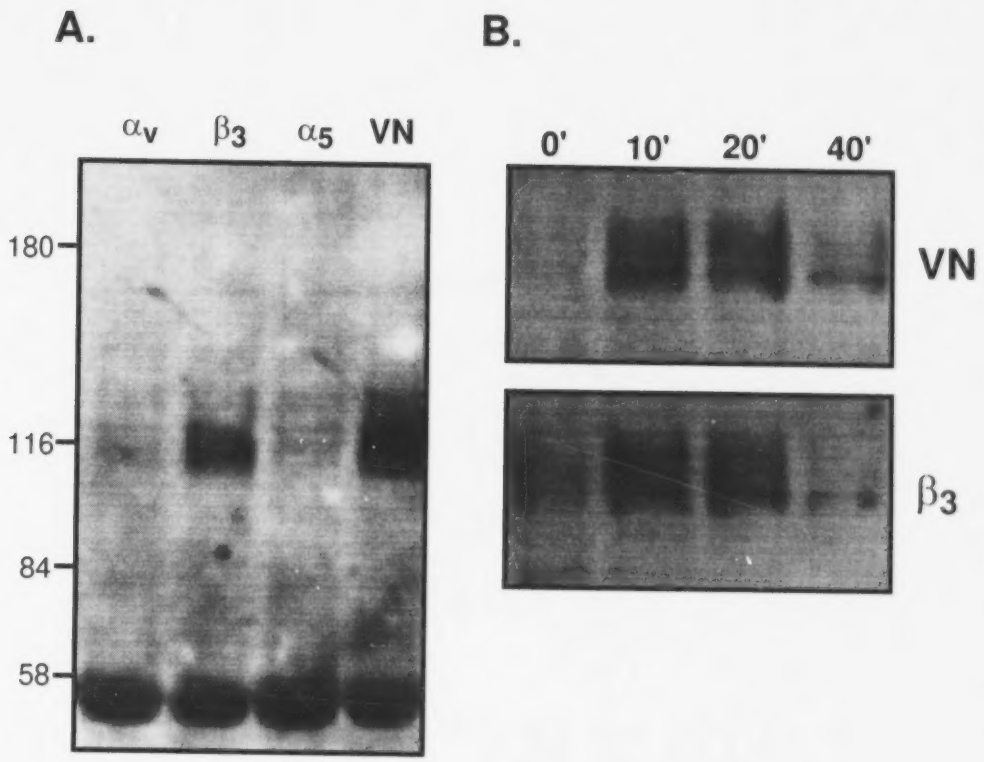

FIGURE 14. Integrin antibody stimulation of tyrosine phosphorylation. Antibodies to the various integrin subunits at 20 μg/ml or VN at 10 μg/ml were immobilized to microtitre plate wells. 10^5 CTL clone 11 cells were incubated for A. 20 minutes or B. 0 to 40 minutes. Results are anti-phosphotyrosine blots. β_3 is the IgG mAb. A no antibody lane did not exhibit any phosphoproteins in the 116 kDa range.

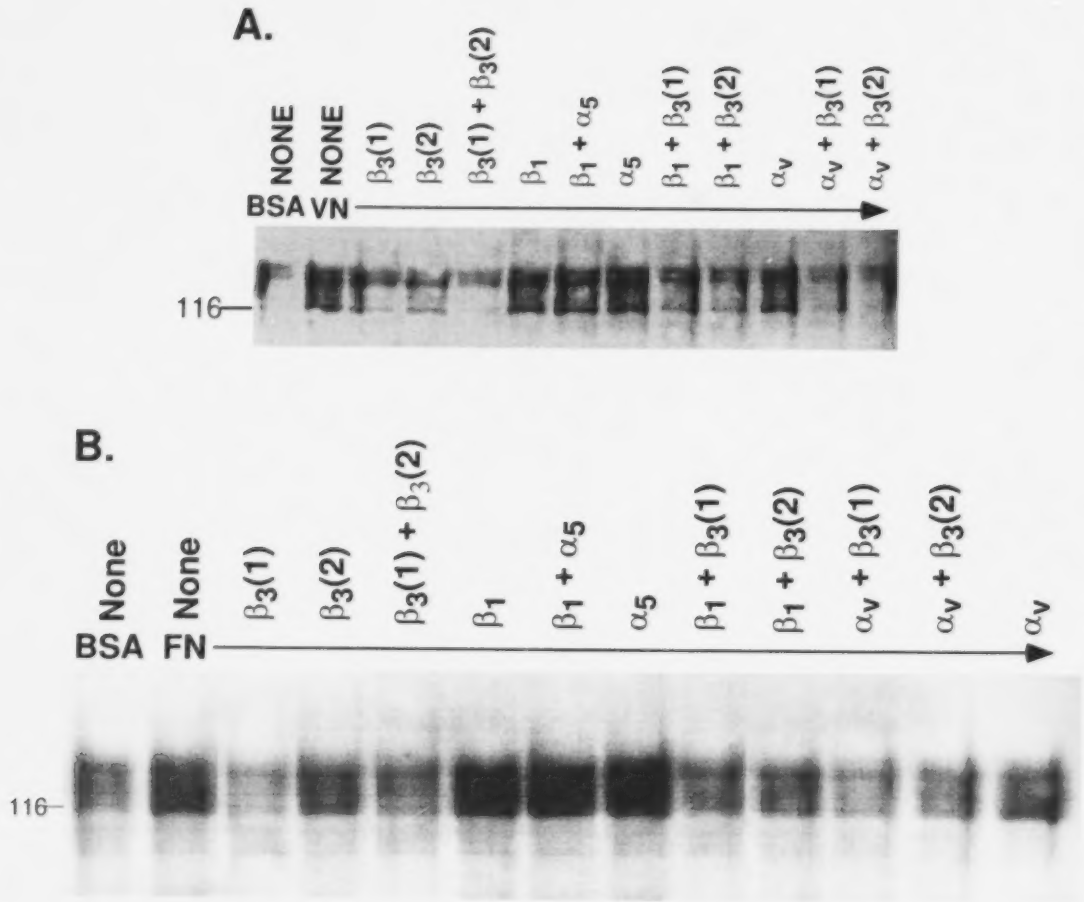

FIGURE 15. Antibody inhibition of VN- and FN-induced tyrosine phosphorylation. CTL clone 11 cells (10^5) were incubated with the indicated integrin antibodies at 10 μg/ml prior to addition of cells to microtitre wells coated with 10 μg/ml of A. VN or B. FN. $\beta_3(1)$ is the IgM mAb and $\beta_3(2)$ is the IgG mAb. Cells were incubated in the wells for 20 minutes. Results are anti-phosphotyrosine blots.

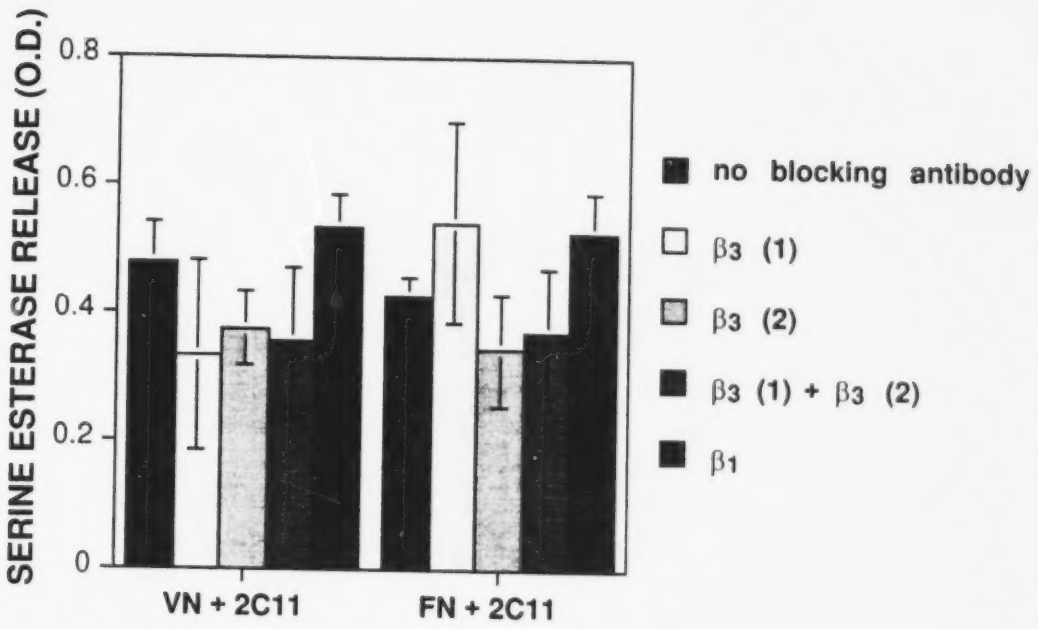

FIGURE 16. Antibody inhibition of VN- and FN-facilitated degranulation stimulated by anti-CD3. Microtitre plate wells were coated with VN or FN at 10 μg/ml co-immobilized with 1 μg/ml 145-2C11. 10^5 CTL clone 11 cells were incubated with the indicated integrin antibodies at 10 μg/ml prior to addition of the cells to the wells. β_3 (1) is the IgM mAb and β_3 (2) is the IgG mAb. Cells were incubated in the wells for 4 hours and assayed for serine esterase release.

the serine esterase release, there was sufficient time for the cells to compensate for adhesion to the substrate by utilizing different integrin receptors.

Identification of the Tyrosine Phosphorylated Proteins

We have observed the increase in phosphorylation of a set of proteins of 115-125 kDa mediated by a β_3 integrin when cells are stimulated with FN or VN. Our next attempt was to identify these proteins. We started by choosing candidate proteins in this molecular weight range that have a role in integrin-mediated signaling. β_3 integrins have been shown to induce the phosphorylation of $pp125^{FAK}$ in platelets[37], and β_1 integrins mediate the CD3-induced phosphorylation of $pp125^{FAK}$ in human $CD4^+$ PBL[79]. However, an antibody to $pp125^{FAK}$ did not immunoprecipitate proteins with enhanced phosphorylation from dendritic epidermal T cell (DETC) lines incubated on FN[83]. When we used the same antibody as the DETC studies, we were unable to immunoprecipitate $pp125^{FAK}$ (data not shown). However, we obtained a different source of antibodies to $pp125^{FAK}$, and this antibody immunoprecipitated a protein that became phosphorylated upon VN or immobilized anti-β_3 stimulation (Figure 17). This protein ran lower that the 125 kDa FAK protein, but corresponded to the major phosphoprotein, pp116, that we have observed. It is possible that this protein is a novel lower molecular weight isoform of $pp125^{FAK}$, or it may in fact not be $pp125^{FAK}$ but a related family member.

We next performed similar immunoprecipitation experiments with antibodies to other proteins in the 115-130 kDa range to possibly identify any other proteins which exhibit enhanced phosphorylation in response to VN or β_3 integrin

engagement. C-cbl is a 120 kDa protein that becomes phosphorylated upon engagement of the TCR complex[93]. We were unable to detect an increase in c-cbl phosphorylation after stimulation through β_3 integrin even though we were able to observe an increase after anti-CD3 stimulation (Figure 18). P120 is a substrate for src and vinculin is a cytoskeletal protein, but the proteins we immunoprecipitated were not of the appropriate molecular weights to be classified in the 115-125 kDa range (data not shown). We have thus far only been able to identify $pp125^{FAK}$ as a protein in the 115-125 kDa size range that demonstrates an increase in phosphorylation mediated by β_3 integrin.

Involvement of the Fyn and Lck Tyrosine Kinases

Since there is an increase in the phosphorylation of several proteins observed after integrin stimulation, we wanted to determine which protein tyrosine kinase(s) may be mediating these events. Two candidates were lck and fyn which have important roles in T cell activation. To test these possibilities, we performed phosphorylation experiments with CTL clones which were negative for either lck or fyn. Stimulation of phosphorylation of the 115-125 kDa proteins by VN or immobilized anti-β_3 was unaffected by the absence of lck (Figure 19A.) or fyn (Figure 19B.). Lck and fyn are both src family kinases, and it has been suggested that a deficiency of one src-related kinase can be compensated for by another since the fyn knockout mouse does not display an overt phenotype[94,95]. Compensation of kinase activity may be occurring in the CTL clones, but it is also possible that neither of the src family kinases are involved and an entirely different kinase is activated.

pp125^{FAK} has been identified as one of the proteins phosphorylated by VN through β_3 integrin. As pp125^{FAK} is itself a kinase and its activation has been correlated with its phophorylation[37], it may be responsible for mediating some of phosphorylation events, including its own phosphorylation. At this point, though, we have not definitively identified any kinases involved in β_3 integrin-dependent stimulation of CTL.

Possible Regulation of β_3 Integrin

The cytoplasmic tail of the β_3 integrin contains a NPXY sequence motif that has been shown to be important in regulating cell migration and integrin binding affinities[96,97]. An NPXY sequence is also found in growth factor receptors to be phosphorylated and capable of recruiting SH2-containing signaling molecules[98]. Since β_3 integrins contain this putative phosphorylation site, we investigated whether the β_3 integrin of the CTL clones was regulated by phosphorylation of the cytoplasmic tail. We immunoprecipitated β_3 from surface biotinylated cells with the IgG mAb, subjected the immunoprecipitate to reducing SDS-PAGE, and assayed for tyrosine phosphorylation by immunoblotting. Integrin β_3 and an α chain were immunoprecipitated as shown by blotting with streptavidin conjugated to horseradish peroxidase (Figure 20A.). However, there was no indication of phosphorylation of β_3 integrin when the same gel was immunoblotted with anti-phosphotyrosine, whether the cells were taken straight from culture or stimulated with VN, even though enhanced phosphorylation of the 115-125 kDa proteins was seen in a sample of the lysate (Figure 20B.).

In platelets, β_3 integrin can be found in association with a 50 kDa integrin-associated protein (IAP). IAP has been implicated in the regulation of ligand binding of $\alpha_v\beta_3$ integrin[99]. As such, we wanted to determine if β_3 on CTL associated with any other proteins. Immunoprecipitation of β_3 integrin with 1% NP40 (Figure 20C.) or gentle detergents, 1% digitonin and 0.5% NP40 (data not shown), did not uncover any biotinylated cell surface molecules coimmunoprecipitating with β_3. Also, we did not observe any tyrosine phosphorylated proteins which coimmunoprecipitated with β_3 (Figure 20B.)

An Intact Cytoskeleton is Required

Studies of integrin-mediated signaling have implicated the necessity of an intact cytoskeleton in the cell's ability to transduce the signals[37,66]. To determine if an intact cytoskeletal structure was required in CTL for integrin-dependent signal transduction, we incubated the cells with cytochalasins, which prevent actin polymerization, prior to incubation with stimulatory ligands. In a dose-dependent manner, both cytochalasin D (Figure 21) and E (Figure 22) inhibited tyrosine phosphorylation of the 115-125 kDa proteins in response to FN (Figure 21A. and 22A.) and VN (Figure 21B. and 22B.) in the presence or absence of sub-stimulatory anti-CD3. This result demonstrates the importance of the cytoskeleton in signal transduction when cells encounter ECM.

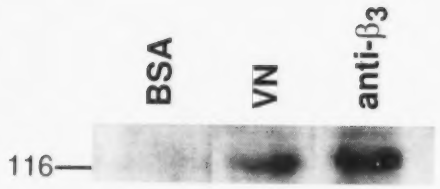

FIGURE 17. Immunoprecipitation of a pp125^{FAK}-reactive protein phosphorylated by stimulation with VN or β_3 integrin antibody. CTL clone 11 (7.5 x 10^6) were incubated in petri dishes coated with BSA, 10 μg/ml VN, or 20 μg/ml immobilized anti-β_3 (IgG mAb) for 30 minutes, lysed in 1.5% NP40 + 1 mM vanadate, and postnuclear lysates immunoprecipitated with anti-pp125^{FAK}. The immunoprecipitate was subjected to SDS-PAGE and immunoblotted with anti-phosphotyrosine.

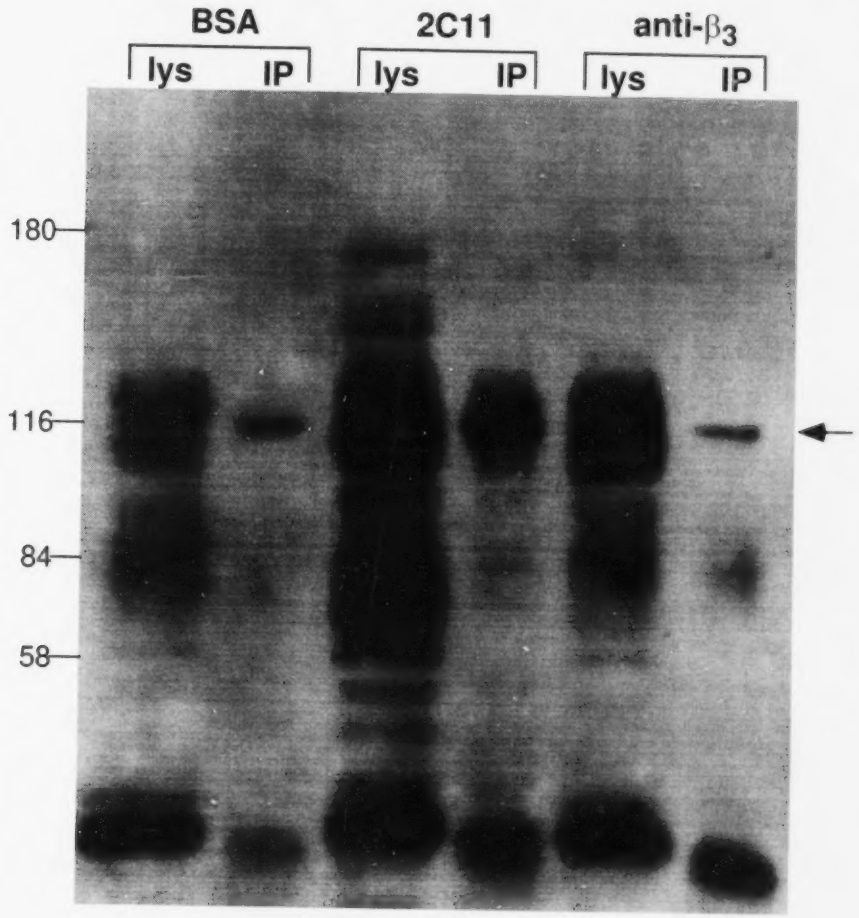

FIGURE 18. C-cbl is present but not tyrosine phosphorylated by immobilized β_3 antibody in CTL clone 11. 7.5 x 10^6 cells were incubated in petri dishes coated with BSA, 12.5 μg/ml 145-2C11, or 20 μg/ml anti-β_3 (IgG mAb) for 30 minutes, lysed, and postnuclear lysates immunoprecipitated with c-cbl antibody. The immunoprecipitate was electrophoresed and immunoblotted with anti-phosphotyrosine. The arrow indicates the position of c-cbl.

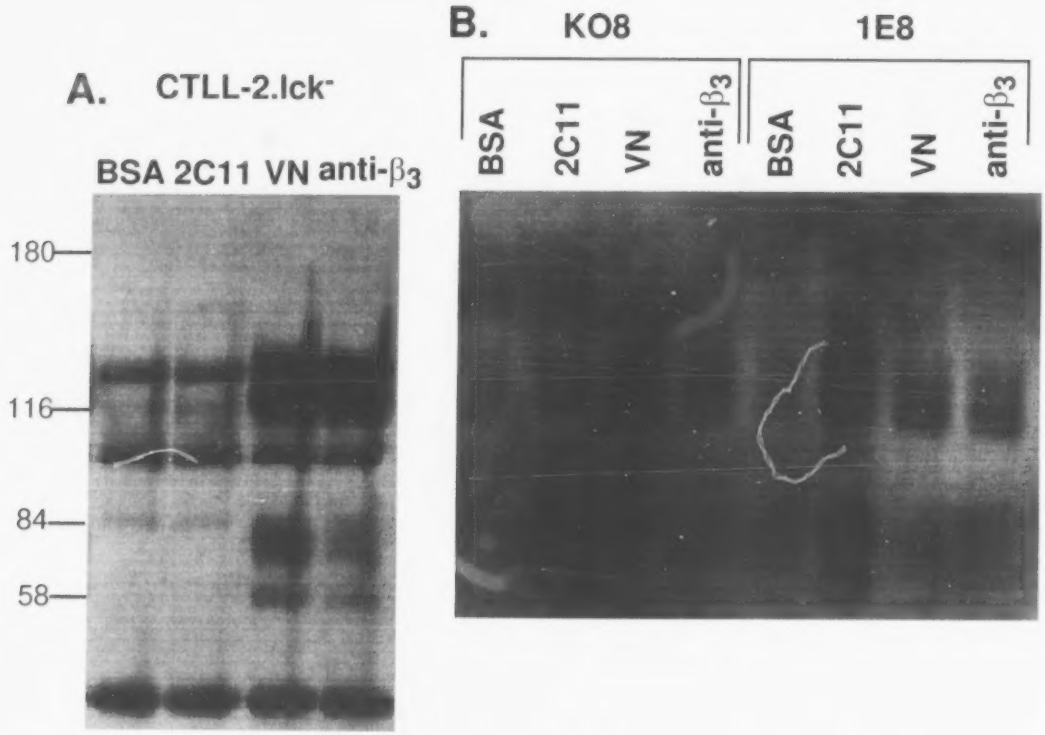

FIGURE 19. Induction of tyrosine phosphorylation in lck-deficient and fyn-deficient CTL clones. A. CTLL-2.lck$^-$, an lck-negative CTL clone, or B. KO8 and 1E8, two different fyn-negative CTL clones were plated at 10^5 cells per well for 20 minutes in microtitre plate wells immobilized with BSA, 20 μg/ml 145-2C11, 10 μg/ml VN, or 20 μg/ml anti-β_3 (IgG mAb). Results are anti-phosphotyrosine blots.

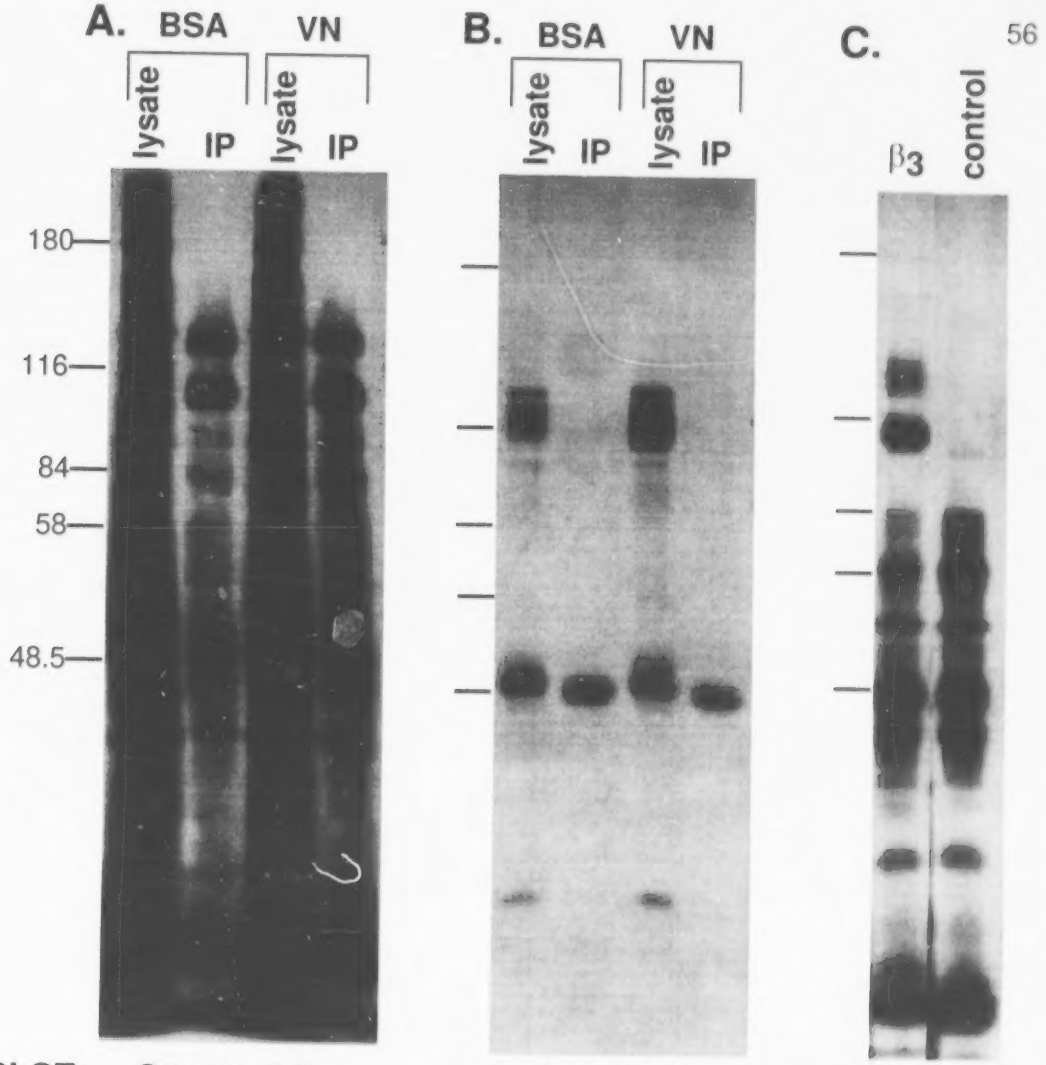

FIGURE 20. Examination of the tyrosine phosphorylation and coimmunoprecipitated proteins of β_3 integrin. CTL clone 11 were surface biotinylated and incubated in BSA- or VN (10μg/ml)-coated petri dishes at 7.5 x 10^6 cells per dish for 30 minutes and lysed (A. and B.), or 2 x 10^6 cells were lysed without stimulation (C.). β_3 integrin was immunoprecipitated with anti-β_3 (IgG mAb), and the immunoprecipitates were electrophoresed and blotted with streptavidin conjugated to horseradish peroxidase (A. and C.). B. is an anti-phosphotyrosine blot of A.

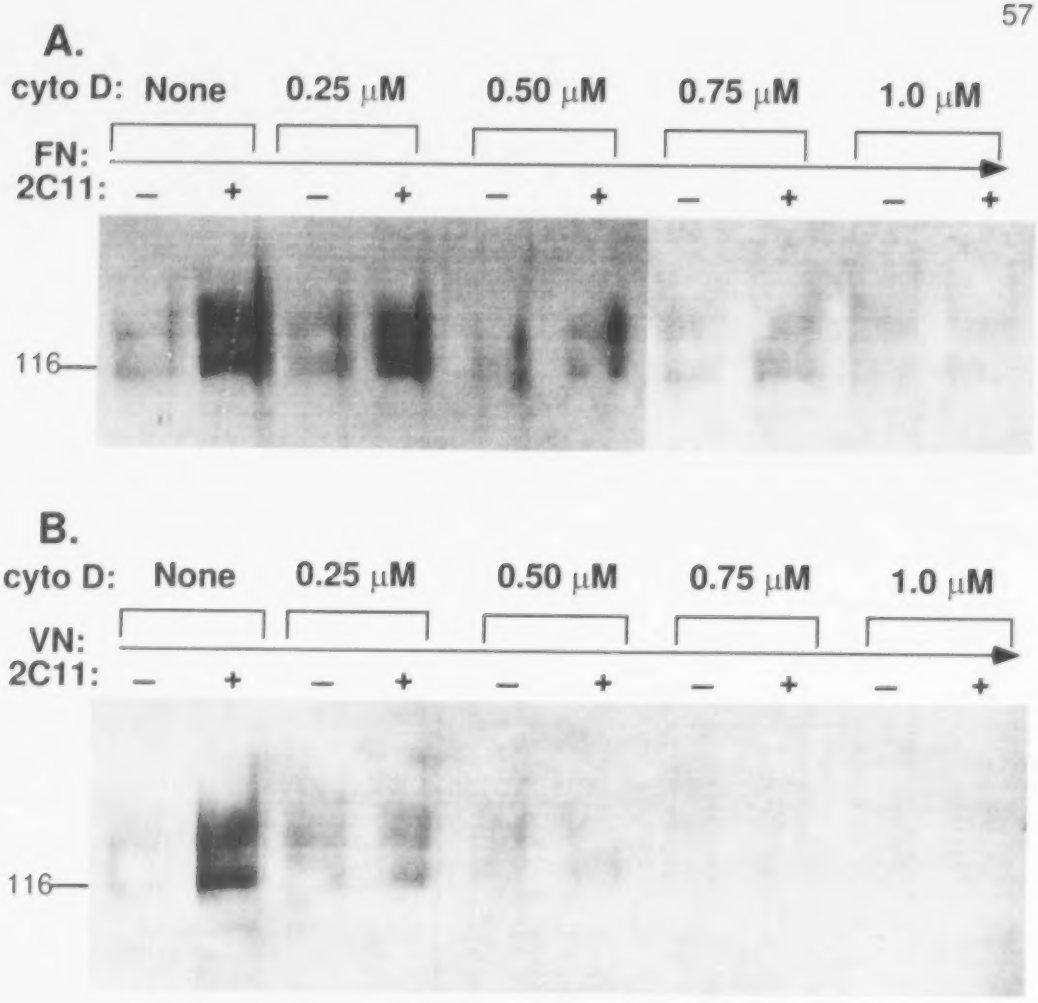

FIGURE 21. Cytochalasin D inhibition of tyrosine phosphorylation induced by FN or VN in the presence and absence of anti-CD3. Wells of a microtitre plate were coated with 10 μg/ml of FN (A.) or VN (B.) in the presence or absence of 1 μg/ml 145-2C11. The indicated concentrations of cytochalasin D were incubated with CTL clone 11 prior to addition of 10^5 cells to each well for 20 minutes. Results are anti-phosphotyrosine blots.

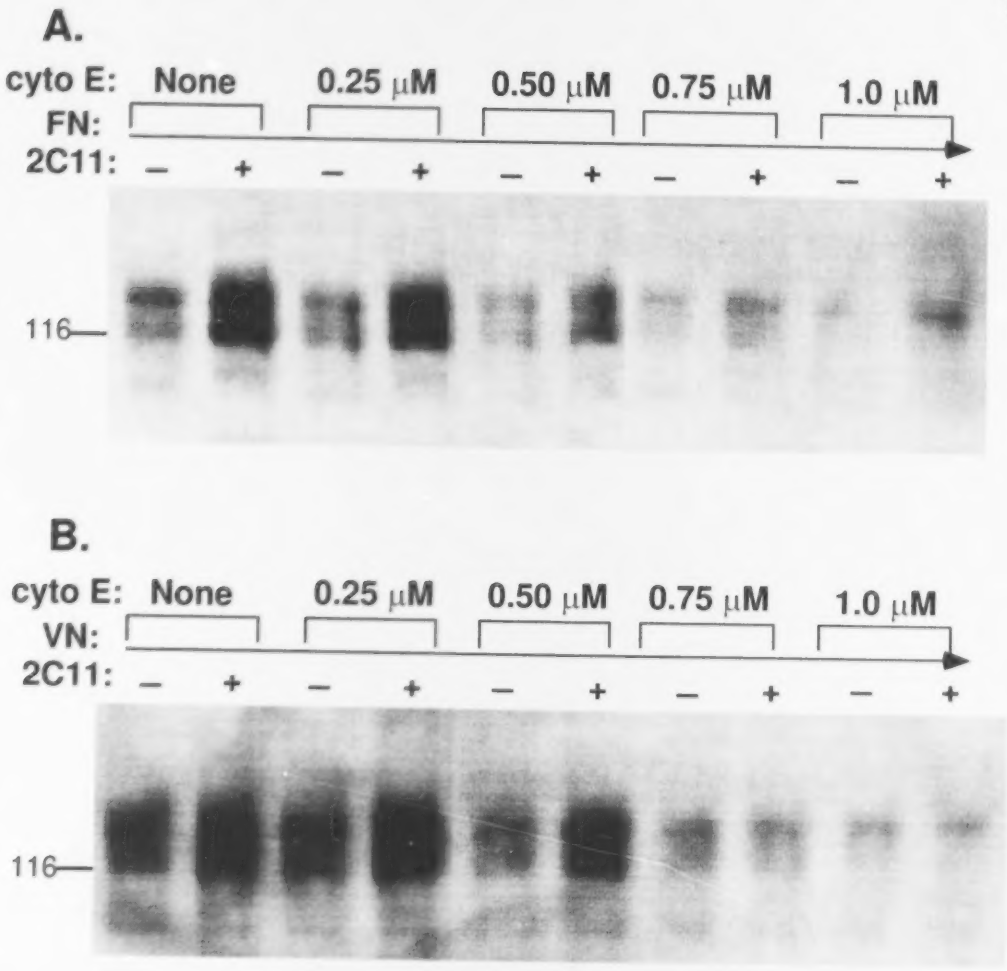

FIGURE 22. Cytochalasin E inhibition of tyrosine phosphorylation induced by FN or VN in the presence and absence of anti-CD3. The same procedure as in Fig. 21 was followed, except with cytochalasin E.

CHAPTER IV.

DISCUSSION

A β_3 integrin Mediates Costimulation

We have shown that FN and VN are capable of facilitating the degranulation of CTL when encountered with sub-stimulatory amounts of anti-CD3. Also, these ECM proteins are able to induce the tyrosine phosphorylation of proteins of 115-125 kDa in these antigen-driven, IL-2-dependent, α/β^+ TCR, $CD8^+$ CTL clones. The specificity of the responses suggested that a β_3 integrin, in particular, the vitronectin receptor ($\alpha_v\beta_3$) may be mediating the interactions. Through FACS analysis and immunoprecipitations, we have generated evidence that the VNR is expressed on the CTL. Direct evidence implicating a functional β_3 integrin is the observation that immobilized antibodies to β_3 can stimulate the phosphorylation of the 115-125 kDa proteins. The β_3 antibodies added in soluble form to the cells are able to inhibit the phosphorylation of the major protein seen in this range, pp116, inducible by FN or VN. The VNR has been shown to be an RGD-dependent integrin[16], and we are able to block degranulation and phosphorylation with a RGD-containing peptide and not with a control RGE-containing peptide.

We have clearly demonstrated the expression and function of a β_3 integrin in CTL, and there is some evidence that α_v is associating with β_3. It is possible, however, that another α subunit chain may be heterodimerized with β_3 in CTL. At present, there have been only two α chains found in association with β_3. α_v and β_3 form the VNR, and α_{IIb} and β_3 form the platelet glycoprotein IIb-IIIa. There may be

additional, as yet undiscovered, α subunits that could function in association with β_3.

The VNR has been studied in lymphoid cells, although not as extensively as the VLAs. The studies which concentrated on CTL are scarce, with results that demonstrated FACS reactivity to a VNR antibody[28]. A subset of γ/δ^+ TCR T cell lines have been shown to express the VNR and utilize it as an accessory molecule for the activation of cytokine secretion[81]. Resting murine spleen, lymph node, and thymus cells do not express VNR, and low levels of expression are inducible only after Con A activation of spleen cells[28]. However, the VNR is found on about 25% of human peripheral blood T cells in the absence of stimulation[72]. Natural killer cells exhibit tyrosine phosphorylation and facilitated cytokine production and proliferation when the VNR is engaged[100].

Differences with Previous Studies

Several studies done on $CD4^+$ human peripheral blood T cells have shown that the β_1 integrins VLA-4 and VLA-5 are responsible for mediating all the FN-stimulated proliferative responses[69,70,74,75,76]. β_3 integrins do not appear to have a role, in that $CD4^+$ PBL were not costimulated with VN and only weakly with fibrinogen[75]. Our studies are done with $CD8^+$ T cells, but the differences observed between our studies and those using $CD4^+$ T cells is most likely not a CD4/CD8 difference. We have demonstrated expression of a β_3 integrin on a murine $CD4^+$ T cell clone and the ability of VN to stimulate phosphorylation of the 115-125 kDa proteins in two $CD4^+$ T cell clones. We do not believe that these results are due to an artifact of species differences as there is expression of VNR on human PBL[72].

It is much more likely that the differences reflect the activation state of the cells used in the experiments. We are using chronically stimulated T cells whereas the $CD4^+$ PBL were unstimulated, resting cells. Consistent with this, resting cells from murine lymphoid tissues do not express the VNR, and its expression in splenic cells is induced only after Con A stimulation[28]. The difference in activation state and responsiveness to multiple ECM proteins may reflect a situation in chronic inflammation. Inflammatory sites, such as the synovium of individuals with rheumatoid arthritis, have significantly higher levels of FN than healthy individuals[101,102]. Also, lymphocytes localized to these inflammatory sites have properties of activated cells[103,104]. The increased levels of ECM proteins may be attracting lymphocytes to inflamed areas. Here, contact with the ECM can enhance events which lead to T cell activation when the appropriate antigen is encountered. Sustained levels of ECM may potentiate a state of chronic inflammation by continually providing stimulation to the cells. There has been a report where FN and VN independently were able to trigger effector functions of T cell clones[85], in contrast to our results, but lending to the possibility of antigen-independent activation of T cell functions by ECM.

Another difference between the cells used in our studies and the others is the site from which the cells were derived. Location in the body may be a regulating factor in the expression of the VNR. We are using cell lines derived from either peritoneal exudate lymphocytes or from spleen. The human studies used cells obtained from peripheral blood. Lymphocytes are highly motile cells with motility being an important aspect of their life cycle, in birth, development, homing abilities, and migration throughout most of the body. Expressing adhesive receptors for ECM proteins does not seem advantageous when cells are traveling

in the circulating blood flow. On the other hand, residence in lymphoid tissues requires adhesion to the extracellular milieu. Migration through tissues also requires transient contacts with the ECM as a substrate on which to "crawl" in order to reach the site of infection or inflammation. In the migratory process where T cells are recruited to such sites, certain interactions with ECM proteins may prime the cells for activation once the appropriate antigen is encountered. Also, for mature, antigen-experienced cells which require less stimulation for activation than naive cells, ECM costimulation may be sufficient or nearly sufficient for activation.

FN has a Dual Role

The mechanism through which FN acts as a costimulatory accessory molecule may simply be due to its adhesiveness which would allow increased cell surface contact with the substrate and, in turn, increase TCR/CD3 engagement such that the signals transmitted through the TCR would reach levels above the threshold for activation. To examine this possibility, a remote signaling assay was done with FN coated wells and anti-CD3 coated latex beads. Since FN and anti-CD3 are on separate surfaces, enhanced effects mediated by FN cannot be due to adhesion that acts to increase TCR/CD3 occupancy. Our results showed that FN was still able to enhance anti-CD3 stimulated degranulation when presented on a different surface[105], in support of a role in signal transduction for FN. However, FN did not increase the maximal amount of degranulation when stimulatory amounts of anti-CD3 beads were used, in contrast with co-immobilized FN and anti-CD3 where FN clearly enhances degranulation over the wide range of anti-CD3 used. This suggests that FN also has an adhesive function in addition to a signaling role that together maximally amplifies TCR signals.

Our remote signaling results are in agreement with Davis et al[70]. Shimizu et al found contradicting results and were unable to costimulate proliferation of $CD4^+$ PBL when ECM proteins and anti-CD3 were presented on two different surfaces[75], indicating that FN is a more effective costimulator when in close proximity to the TCR complex. FN and VN can be expressed on the surface of cells where they can act as ligands for integrin receptors on the apposing cell. Antigen presenting cells may express these ECM proteins on their surface and utilize them in costimulation when presenting to a T cell. Consistent with this hypothesis, antibodies to FN can block recognition of FN on target cells and inhibit lymphokine-activated killer cell cytotoxicity[84]. In these situations, FN would be presented on the same surface as the antigen to function in a dual role augmenting the adhesion between cells and the signals transduced.

Possible Role of the FNR

We have demonstrated a functional β_3 integrin on the CTL, but there is the possibility that the FNR (β_1 integrin) may have a role in the costimulation with FN. The cells do express the α_5 and β_1 subunits. On CTL, the interaction with FN may be mediated by both VNR and FNR with redundant functions. Studies in other cell types indicate that β_1 and β_3 integrins have the potential to respond in different capacities. In smooth muscle cells, β_1 integrins function in adhesion to FN, laminin, and types I and IV collagens, whereas $\alpha_v\beta_3$ is utilized in migration over these ECM proteins[106]. It would be interesting to investigate the possibility that the FNR and VNR have distinct functions in adhesion and migration, respectively, in CTL.

If the VNR and FNR are both involved in costimulation of CTL, they could be functioning independently, or there could be co-operation between them. The FNR collaborates with the VNR through an unknown mechanism in Chinese hamster ovary cell variants in migration on vitronectin[107]. Moreover, antibody ligation of transfected $\alpha_v\beta_3$ blocks phagocytosis mediated by $\alpha_5\beta_1$ in the erythroleukemia cell line K562[108]. The expression of multiple integrins may each contribute distinct and subtle functions which are integrated and utilized by the cell to attain a desired outcome.

Besides integrins, there are countless other accessory molecules present on the surface of a T cell. Each accessory molecule, as well, has its own function. Some overlap with others, and the redundancy could be the amplification required for activation. FN and VN, through a β_3 integrin, appear to activate the same signaling pathways as the TCR that results in the tyrosine phosphorylation of the same substrates. Still others, e.g. CD28 and B7, have entirely different signaling capacities[11]. Trying to identify the contributions of individual accessory molecules requires isolating the signaling pathways and proteins which are regulated by them.

Tyrosine Phosphorylation of a pp125^{FAK}-Reactive Protein

We have observed that β_3 integrin engagement activates kinase activity and tyrosine phosphorylation of several proteins in the molecular weight range of 115-125 kDa. One protein in this cluster reacts with an antiserum specific for pp125^{FAK} and becomes phosphorylated upon stimulation with VN or immobilized anti-β_3. Several other mAb and polyclonal antisera to pp125^{FAK} were unable to

immunoprecipitate a similarly inducible protein. The CTL, perhaps, express a FAK-related molecule that cross-reacts with the antiserum we used. FakB is a lymphocyte-specific form of pp125^{FAK}[54], and CAKβ is a FAK family member that is localized to areas of direct cell-cell contact[55]. CAKβ could be more relevant in CTL interactions with a target cell, where there is tight juxtaposition of the two cells and, potentially, focal adhesion formation at the contact points of the cells prior to lysis. Also, CAKβ is a smaller protein than pp125^{FAK}, running at an apparent molecular weight of 113 kDa[55]. The pp125^{FAK}-reactive protein we immunoprecipitated is 116 kDa, smaller than pp125^{FAK} in adherent cells.

Tyrosine Phosphorylation of Additional Proteins

The FAK-related protein we have isolated is one of several proteins of 115-125 kDa that becomes phosphorylated in response to FN and VN. It will be important to identify these other proteins and determine if they or the FAK protein are directly involved in the downstream triggering of degranulation facilitated by FN and VN in response to anti-CD3. The same conditions that stimulate phosphorylation of the CTL also facilitate degranulation, suggesting the two events are linked, such that the β_3 integrin mediates signal transduction of some, as yet unknown, pathways leading to degranulation. Protein tyrosine phosphorylation is an early signal transduction event and degranulation is a late event. It will be worth investigating what events downstream of phosphorylation are affected by β_3 integrin engagement. It has been demonstrated that CTL adhesion to FN enhanced phosphoinositide hydrolysis initiated through the TCR[77].

Tyrosine Kinases are Activated and Mediate Phosphorylation

The phosphorylation of the proteins we observe are mediated by tyrosine kinases which are activated with FN and VN stimulation . The src-related kinases lck and fyn were likely candidates that functioned in this capacity, since they are important kinases in T cell activation. However, an lck-negative CTL clone and two fyn-negative CTL clones are capable of inducing phosphorylation of the 115-125 kDa proteins. The deficiency of either lck or fyn does not inhibit phosphorylation, but it is possible that the deficiency of one of the src-like kinases is compensated by another. This possibility is illustrated by the $fyn^{(-/-)}$ mouse which has no overt phenotype[94,95]. Lck and fyn may still be important for phosphorylating a subset of focal adhesion proteins. $pp125^{FAK}$ or a FAK-related protein tyrosine kinase may also have a role in integrin signaling by mediating autophosphorylation and phosphorylation of additional substrates. On the other hand, an entirely different kinase may be involved in phosphorylation of the 115-125 kDa proteins. Tyrosine phosphorylation is a common regulatory mechanism of protein function and promotes signal transduction. Protein phosphorylation is implicated in regulating the formation of focal contacts of a migratory T cell or a CTL conjugated to a target cell. Pathways regulated by phosphorylation may also direct signals to promote other functions such as degranulation.

Role of the Cytoskeleton

We have shown that treatment of the CTL with cytochalasins, which disrupt the network of actin microfilaments, inhibits the phosphorylation of proteins in response to FN or VN alone and when co-immobilized with anti-CD3. This result

demonstrates the importance of an intact, dynamic cytoskeleton to permit tyrosine phosphorylation events to occur. Cytoskeletal components form the cytoplasmic face of focal adhesion contacts, and blocking actin polymerization would, thus, disrupt the formation of focal contacts. It is conceivable that the cytoskeleton also serves as scaffolding to recruit proteins and position them correctly to catalyze their reactions. If the tyrosine kinases involved in phosphorylation in focal adhesions depend on the cytoskeleton for localization, then disruption of the cytoskeleton would adversely affect the ability of the kinases to function.

An Elaborate Integrin System

There have been many studies done on integrin expression and function on lymphoid cells. They have illustrated a complex regulation of expression and participation in numerous cellular activities. In lymphocytes, adhesive interactions with the extracellular matrix, found throughout tissues and on cell surfaces, have important outcomes throughout the life cycle of the cell. Migration and circulation throughout the body is dependent on transient adhesion and can lead a T cell to a specific location. Arrest at a particular site exhibits more tight adhesion and formation of stable focal contacts. These interactions are probably largely regulated by the multitude of integrins that a T cell can potentially express. It is possible that the integration of signals mediated by integrins and generated by contact with ECM is under intricate cellular regulation with the many other receptor signaling pathways, which develops a highly complex regulatory network for T cells that controls adhesion and effector function.

CHAPTER V.

CONCLUSIONS AND FUTURE DIRECTIONS

Several conclusions can be drawn from our experiments. First, we have demonstrated the expression of β_3 integrin on CTL. Direct stimulation through the β_3 integrin with immobilized antibodies to β_3 can trigger the tyrosine phosphorylation of several proteins of 115-125 kDa. The time course of the transient phosphorylation is the same as when the CTL are incubated on β_3 integrin ligands, FN and VN. FN and VN can also induce the phosphorylation of the 115-125 kDa proteins, and the phosphorylation is enhanced and prolonged when co-immobilized with sub-stimulatory amounts of anti-CD3. The phosphorylation events are dependent on an intact cytoskeleton of the CTL.

We have identified a 116 kDa protein, recognized by an antibody generated against $pp125^{FAK}$, that becomes tyrosine phosphorylated upon VN stimulation or β_3 integrin engagement. This $pp125^{FAK}$-reactive protein is only one of the 115-125 kDa proteins that becomes phosphorylated under the same conditions.

As well, we have shown that FN and VN are able to facilitate degranulation of CTL in response to sub-stimulatory anti-CD3. Clearly, there is a costimulatory role for FN and VN in CTL activation and subsequent degranulation and a β_3 integrin which mediates early signaling events. The same conditions that facilitate degranulation also stimulate tyrosine phosphorylation, suggesting that the two events are linked. We were able to inhibit degranulation and tyrosine phosphorylation with RGD peptides. β_3 integrin antibodies were effective at

blocking tyrosine phosphorylation of pp116, a major phosphoprotein, but we were unable to significantly block degranulation with anti-β_3. It still remains to be seen if the β_3 integrin is responsible for the signals which lead to degranulation. The signaling events downstream of phosphorylation and upstream of degranulation are as yet unidentified.

We were able to induce tyrosine phosphorylation stimulated by VN and immobilized anti-β_3 in lck- or fyn-deficient CTL clones, suggesting the absence of lck and fyn tyrosine kinases did not affect the tyrosine phosphorylation events. However, there is a possibility that the deficiency of one of the src-related kinases is compensated for by the activity of another src-related kinase. Members of the src family of tyrosine kinases can localize to focal adhesions through association with FAK and may still mediate the phosphorylation of some proteins. $pp125^{FAK}$ has tyrosine kinase activity, as well, which may autophosphorylate in the CTL clones or be responsible for the phosphorylation of some of the proteins which we detect. Alternatively, an entirely different kinase may function in this capacity.

One of the proteins in the 115-125 kDa range is recognized by a $pp125^{FAK}$ antibody, but it could be actually be a lower molecular weight isoform of $pp125^{FAK}$ or a FAK-related member such as fakB or CAKβ. In the future, the availability of reagents for these proteins will greatly aid in distinguishing the possibilities. Once identified, it would be interesting to visualize the localization of the tyrosine phosphoproteins, whether they aggregate with β_3 integrin in focal adhesions or whether they are signaling molecules discreetly dispersed in the cytoplasm or nucleus. Further research could be done to identify the other 115-125 kDa proteins that are phosphorylated by stimulation with FN and VN through β_3 integrin.

We can detect expression of β_3 and α_v integrin subunits, suggesting the VNR is expressed by the CTL clones. However, the α_v antibody was not reliably effective such that we cannot definitively identify the β_3 integrin as the VNR as we speculate it to be. There is a lack of available antibodies to murine integrins. A β_1 integrin antibody that recognizes $\alpha_5\beta_1$ would be extremely useful. We could then approach uncovering the possible contribution of the FNR. It would also be worthwhile to investigate any other integrins which are expressed by the CTL and their potential functions.

We hope that our results have aided in unraveling and understanding integrins, their complexity of expression, and function in costimulation of T cell activation. Integrins are not just merely adhesive receptors, but have important signaling functions in a variety of cell types. Hopefully, continuation of integrin research will allow us to fully appreciate their contributions.

CHAPTER VI.

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